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# Bacillus cereus and Bacillus anthracis germination kinetics: A Michaelis-Menten approach

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# *BACILLUS CEREUS AND BACILLUS ANTHRACIS* GERMINATION KINETICS:

# A MICHAELIS-MENTEN APPROACH

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ABSTRACT

***Bacillus cereus and Bacillus anthracis* Germination Kinetics: A Michaelis-Menten Approach**

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*Bacillus* species are rod-shaped, gram-positive bacteria that are capable of producing endospores. In this dormant stage, the endospores can persist in hostile physical and chemical environments. Once conditions become favorable, the spores germinate into actively dividing cells, vegetative cells. Germination is a crucial step for the pathogenicity of the *Bacilli* in affecting a host organism.

Our study applies mathematical approaches to spore germination to determine whether the binding of one germinant will affect the binding of another germinant. We pursued this approach with two different species, *B. cereus* and *B. anthracis*, both pathogenic organisms. *B. cereus* is a widely known food pathogen that causes food-borne illnesses. *B. anthracis*, anthrax, is most commonly known for the 2001 bioterrorism attacks.

Both *B. cereus* and *B. anthracis* germinate with a variety of amino acids and nucleosides. *B. cereus* was shown to have cooperative effects with inosine and L-alanine induced germination. We studied the effects of inosine and L-alanine germination in response to cooperative binding. We showed that allosteric cooperativity is seen with the inosine and L-alanine binding sites between the GerI and GerQ receptors. With *B. anthracis,* we used 10 different combinations of amino acid and nucleosides to

understand the different pathways of germination response. We suggest a mechanism of binding that requires cooperativity among inosine with L-serine and inosine with L-methionine binding.

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# CHAPTER 1

# INTRODUCTION

## 1.1 The genus *Bacillus*

*Bacillus* is a genus comprised of rod-shaped bacteria that are capable of forming endospores. The name *Bacillus* was first established in 1872 by Ferdinand Cohn. By 1876, Cohn and Koch detected endospores formed within vegetative cells. This ability to form endospore is an important characteristic of bacilli. (1).

Early microbiologists indicated that *Bacilli* are saprophytic organisms whose natural habitat was soil (2). Organic materials are stored in the nutrients in soil and serve as a nutrient for microbial life (5). However, the composition of soil varies widely from location to location. Nevertheless, soil is an important reservoir for *Bacilli,* housing approximately $1.5 \times 10^{10}$ bacteria/gm (3, 4).

The environment of soil is generally harsh for bacterial growth, (6) suggesting that vegetative cells generally do not survive well. However, endospores are able to withstand the adverse conditions that the soil environment presents. Thus, it is unclear how *Bacilli* can withstand and complete its life cycle from germination to re-sporulation in soil. There are studies that indicate that *Bacillus* species does not grow in soil but rather germinate and grow in animal host (7, 8). However, a study indicated that *B. cereus* is capable of undergoing a complete life cycle of growth, germination to sporulation in soil (9).

## 1.2 Endospores

In 1933, Bayne-Jones and Petrilli first demonstrated with photographic evidence the idea of a cell giving rise to one spore (10). The developing spore showed a dense area of protoplasm that resulted in the formation of a mature spore that is followed by its release from the cell. The use of phase contract microscopy also confirms the sporulation pathway in the cell (11).

*Bacillus* species are present in two different physiological states: vegetative cell and the endospore. The bacterial endospore is one of the most resistant and dormant life forms, it can be $10^4$ times more heat resistant than the vegetative form of the same species (12). Spores have a highly dense and structured morphology that contributes to its resistant capacities. Chemical resistance of the spore is dependent on the protection of the spore coat and inner membrane, dehydration of the spore core, and by small, acid-soluble proteins SASP (13).

When specific nutrients are present in sufficient quantities, *Bacilli* cells continue the normal vegetative growth and proliferate. Once these nutrients are depleted, *Bacilli* cells initiate the sporulation program as a response to starvation. When nutrients return to the environment, the *Bacilli* spore germinates and resumes normal vegetative growth. The survival of a spore is dependent on its ability to germinate into a metabolically active growing vegetative cell.

### 1.2.1 Sporulation

Asymmetric cell division is fundamental to the development of multi-cellular organisms, but also in rod-shaped bacteria that undergoes a specific differentiation process termed sporulation. The following is a description of the sporulation process

based on the well studied spore-forming bacterium, *Bacillus subtilis*. The process of sporulation in *B. subtilis* has been extensively reviewed (14, 15, 16, 17). It is expected that other *Bacilli* and *Clostridia* use a similar sporulation program.

The initiation of sporulation is controlled by the transcriptional regulator protein Spo0A (18). The activity of Spo0A is regulated by phosphorylation through a complex network of kinases that transfers phosphate to Spo0A (16). Once the concentration of the phosphorylated-Spo0A reaches a threshold, phosphoylated-Spo0A activates the transcription of sporulation specific genes and the transcription of RNA polymerase sigma factors $\sigma^F$, and $\sigma^E$. In addition to Spo0A, $\sigma^H$ is also required for the initiation of sporulation and regulates the transcription of early sporulation-specific genes, though the activity of $\sigma^H$ is not well understood (19, 20).

The first crucial morphological event in sporulation is an asymmetric cell division. This division causes the cell to divide at a polar site in the cell leading to dissimilar gene expression and different cell fates (21, 16). The division causes the formation of a protoplast or forespore and a mother cell. Asymmetric division triggers the activation of $\sigma^F$ that regulates gene expression in the forespore and $\sigma^E$ that regulates gene expression in the mother cell. The combined action of these two sigma factors triggers the next morphological event; the engulfment of the forespore by the mother cell, pinching it off as a cell within a cell. Upon completion of the engulfment process, two more RNA polymerase sigma factors are activated: $\sigma^G$ and $\sigma^K$. $\sigma^G$ regulates gene expression in the final stages of development in the forespore, including expression of the germination (Ger) receptors. $\sigma^K$ regulates late gene expression in the mother cell that including the spore coat proteins (22). The forespore will eventually become the spore and the mother

cell terminally differentiates and leads to its own lysis and the release of the spore (See Figure 1).

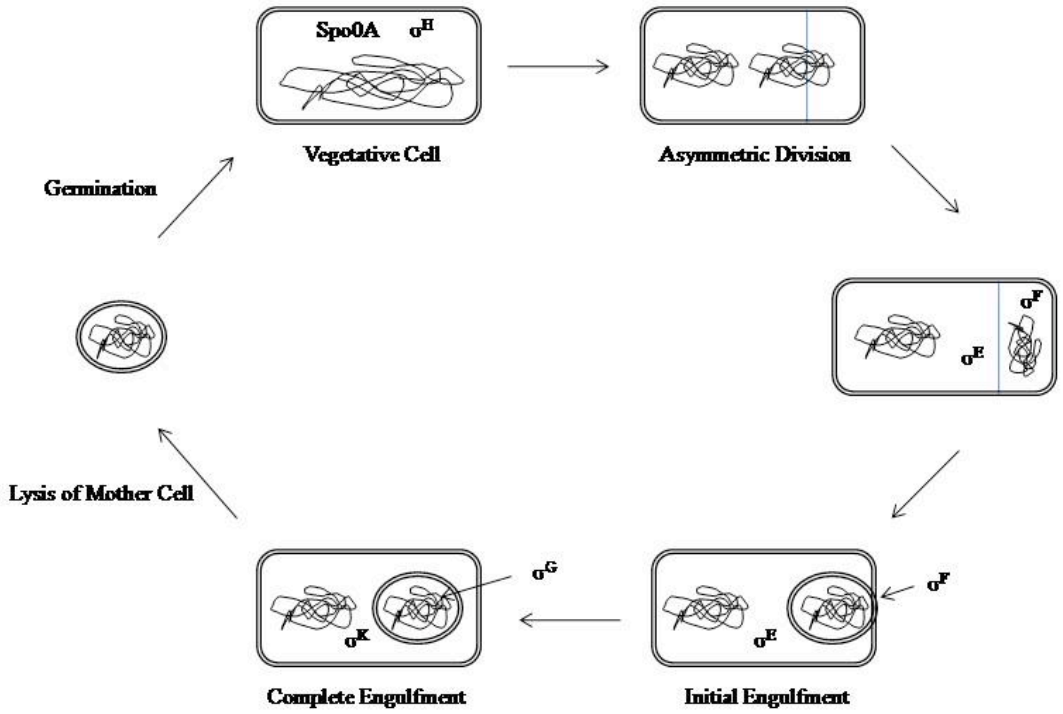



Figure 1. Process of sporulation. Once Spo0A becomes phosphorylated it enters into sporulation. $\sigma^H$ is required for the initiation of sporulation. Vegetative DNA replicates and forms two identical daughter chromosomes and migrates to opposite poles. An asymmetric septum divides the cell into a mother cell and a forespore. After the division $\sigma^F$ and $\sigma^E$ becomes activated. The forespore is engulfed by the mother cell, after the engulfment $\sigma^G$ and $\sigma^K$ becomes activated. The forespore develops into a spore. Consequently, the mother cell lyses and allows for the release of the mature spore.

### 1.2.2 Spore Structure

The bacterial endospore is a complex structure composed of several layers (see Figure 2). The main structural components are detailed below:

Spore Core - The innermost region is the core, which corresponds to the developing forespore. Most of the endospore resistance results from the structure and composition of the core. The spore core eventually becomes the cytoplasm of the germinated cell and hence contains DNA, RNA, and enzymes. However, these biopolymers have to be protected against environmental conditions. During the final stages of sporulation, the core becomes dehydrated. Dehydration has been shown to be a key parameter in the development of resistance by spores (23, 24). It has a very low water content and high levels of dipicolinic acid (DPA) and calcium cations that continue to provide a role in spore resistance (24). The histology of the core remains largely unknown.

Another determinant for resistance in the spore core is the presence of a group of small, acid-soluble proteins (SASP). SASP are proteins of the α/β type and has been shown to be highly conserved amongst *Bacillus* species. These proteins have been shown to protect the spore DNA from heat and UV damage (25).

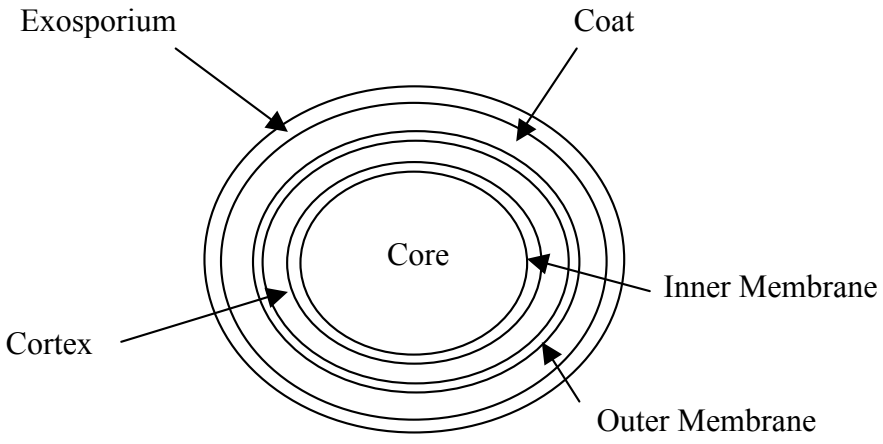



Figure 2. Structure of the endospore.

Inner Membrane - The inner membrane is composed of a polycrystalline structure that surrounds the core of the spore. The inner membrane acts as a selective permeability membrane. Germination receptors are found to be located in this region, thus indicating that the inner membrane is important in the germination process (26). Altering the inner membrane has been seen to affect germination properties (27).

Cortex - The cortex, composed of a thick layer of peptidoglycan, surrounds the inner membrane of the spore. It has been found to be involved in the maintenance of heat-resistant dormant state of the spore (28) and contributes to the dehydrated state of the core (29).

Outer Membrane - The outer membrane is found between the cortex and the spore coat. Its function is unclear, however it is an essential structure formed during sporulation (17).

Spore Coat - The spore coat is a protein shell that constitutes a major part of the spore. The spore coat is a multilayered structure that encases the spore and is composed of many highly cross-linked polypeptide layers (27). The spore coat provides spores with resistance against exogenous lytic enzymes, organic solvents, and oxidative chemicals (29, 30, 31). The spore coat acts primarily as a permeability barrier. The role of the spore coat in germination is unclear, though it does carry a role in spore germination (32, 33).

Exosporium - The exosporium is a glycoprotein layer that surrounds the spore, consisting of a hexagonal crystal-like basal layer and a hairy-nap outer layer (34). The exosporium represents the surface layer that will make initial contact with the host, serving as a semi-permeable barrier for harmful molecules. Currently, the exosporium function is the least understood structure in the spore.

## 1.3 Initiation of Germination

Spore germination is the process in which a spore breaks dormancy and undergoes vegetative growth. In the course of germination, the spore loses its resistant capacities. Despite the spores dormancy and resistance, it is able to detect the presence of specific signaling molecules that initiate the process of germination and outgrowth (35, 36, 37). These signaling molecules, termed "germinants" are comprised of nutrients, chemicals, and physical triggers, such as high hydrostatic pressure (29). Nutrients such as amino acids and nucleosides are believed to induce germination by binding to the germination receptors found in the inner membrane of the spore (38, 39). Once the germinants activate germination receptors, spores are committed to germinate even after the removal of the germinants (40).

Germinants bind to the receptors and trigger the loss of heat resistance and the release of DPA and $Ca^{2+}$ from the core of the spore. This action allows the intake of water resulting in a partly dehydrated core. The replacement with water triggers the hydrolysis of the peptidoglycan cortex, which is important in providing a larger space for the expanding core. This allows more water to enter the spore core. Once the core is completely hydrated, the reactivation of the cell metabolism and macromolecular synthesis occurs (40). The overall effect is the release of the outgrowing spore from the spore coat and the resumption of vegetative growth (40).

## 1.4 Germination Receptors

A family of membrane-bound proteins, germination (Ger) receptors, monitors the environment surrounding the spore for compounds that initiate germination. Initiation of

germination is nutrient specific. The first and best studied model of germination receptors is the *gerA* operon in the genome of *B. subtilis*, which encodes for the GerA receptor (41). The Ger receptor family is composed of tricistronic operons that encode for three gene products necessary to form a functional germination receptor. With the loss of any *gerA* operon homologue, the function of the receptor is eliminated (42, 43, 44).

The tricistronic operon encodes proteins, A, B, and C, of the germination receptor (see Figure 3). Based on hydrophobic plots, the A and B proteins have been proposed to be integral membrane proteins (42, 43). The C protein has been proposed to be relatively hydrophilic with an N-terminal signal peptide for transfer across membranes (41). Localization of the receptor proteins has shown discrepancies. One study concluded that the GerAB protein is located on the outer edge of the cortex (the outer membrane) (45, 46), while another study with *Clostridium* species, a gram-positive spore-former, found that the GerAB receptor was located in the inner membrane (47). Localization of the GerAA and GerAC protein was also found in the inner membrane in *B. subtilis* (39). No other studies have shown the localization of the germination receptors in the outer membrane. Thus the localization of germination receptors are believed to be in the inner membrane.

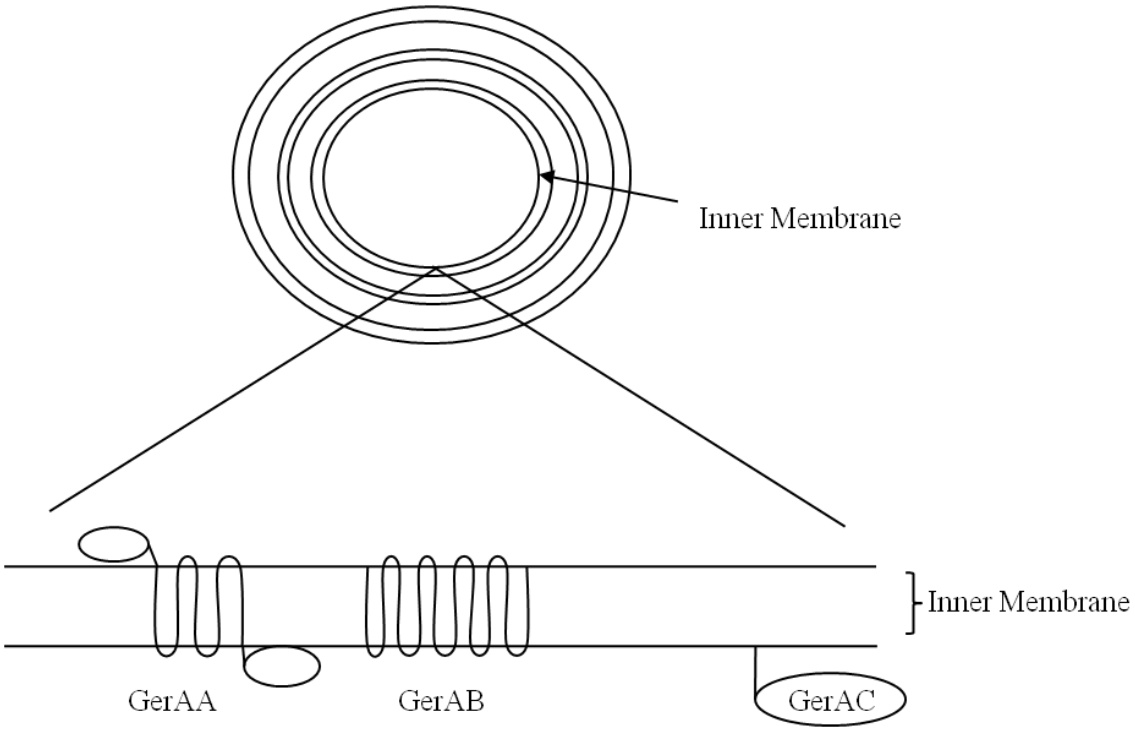



Figure 3. Localization of the receptor complex. Germination proteins A, B, and C are seen in the inner membrane of *B. subtilis*.
Adapted from Moir, A., Corfe, B.M., and Behravan, J. (2002)

## 1.5 *Bacillus Cereus* group

The *Bacillus cereus* group is composed of six closely related species, *B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides*, and *B. weihenstephanensis* (48), the former three are recognized pathogens. *B. cereus, B. anthracis, B. thuringiensis,* and *B. mycoides* share significant degree of genetic similarity based on DNA-DNA hybridization studies (49, 50). It was reported that the four species exhibit greater than 99% similarity in their 16S rRNA base sequences (51).

### 1.5.1 *Bacillus cereus, Bacillus anthracis,* and *Bacillus thuringiensis*

*B. cereus, B. anthracis*, and *B. thuringiensis* have been found to be genetically similar. The genome sequences of *B. anthracis* Ames strain (52), *B. anthracis* Sterne strain (53), *B. cereus* ATCC14579 strain (54), and *B. cereus* ATCC10987 strain (55), and *B. thuringiensis* Al Hakam (56) have been published. Greater than 90% of genetic similarity between *B. anthracis* and both *B. cereus* strains has been reported. Due to their high degree of genetic relationship, it is frequently debated whether *B. cereus, B. anthracis,* and *B. thuringiensis* are members of a single species (57, 58).

*B. cereus, B. anthracis,* and *B. thuringiensis* are the pathogenic species of the *Bacillus cereus* group. *B. anthracis* and *B. thuringiensis* produce toxins that are plasmid borne (54). The key virulence plasmids in *B. anthracis* are pXO1 and pXO2, encoding the anthrax toxin and capsule respectively. *B. thuringiensis* is an insect pathogen, which produces δ-endotoxins (59). *B. thuringiensis* has been used considerably as a biopesticide, for pest control (60). The plasmids carry most of the virulence factors, thus loss of plasmid, *B. anthracis* and *B. thuringiensis* results in loss of virulence.

### 1.5.2 *Bacillus mycoides, Bacillus pseudomycoides*, and *Bacillus weihenstephanensis*

*B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* also belong in the *B. cereus* group, though they are less characterized than *B. cereus, B. anthracis,* and *B. thuringiensis.* They are also capable of forming endospores. *B. weihenstephanensis* is a psychrotolerant bacterium that is capable of growing aerobically at temperatures below 7°C and may form food-borne outbreaks due to the presence of toxin genes such as cereulide (61, 62). *B. mycoides* is an aerobic bacterium that grows at 30°C and appears

nonpathogenic (63). *B. pseudomycoides* is a facultative anaerobic bacterium that grows at an optimal temperature of 28°C. *B. mycoides* and *B. pseudomycoides* can be distinguished from *B. cereus* by colony morphology on agar plates due to their rhizoid shape (64). *B. pseudomycoides* can be distinguished from *B. mycoides* from its fatty acid composition (64).

## 1.6 *Bacillus cereus*

*B. cereus* is a facultative, anaerobic, gram-positive, motile rod-shaped bacterium. The colony morphology on agar plates are large (3-8 nm diameter) and flat with irregular borders (65, 66). *B. cereus* is an endospore forming bacterium that is a common soil saprophyte. In the spore state, the cells are highly resistant to adverse conditions, such as heat, chemicals, and radiation. (13). *B. cereus* has been frequently associated with food spoilage and food-borne illnesses. It secretes a potent necrotizing tripartite enterotoxin called haemolysin BL (67, 68, 69, 70). Two forms of food poisoning have been associated with *B. cereus*, the diarrheal type and the emetic type.

### 1.6.1 *B. cereus* in food products

*B. cereus* food poisoning is found contaminated in the food product and symptoms are seen after its consumption. Both types of food poisoning are mild, however cases in which death occurred has been reported with both emetic and diarrheal types. (71, 72)

### 1.6.2. Toxins

The Emetic Toxin

The emetic type of food poisoning is caused by the emetic toxin found during the growth of cells in food (1). The toxin has been found to be cereulide and consists of a

ring structure with molecular mass of 1.2kDa, composed of three repeats of four amino- and/or oxy-acids (73). Cereulide is hydrophobic and not easily solubilized in aqueous conditions and may be delivered to target cells bound to food products (74). This peptide is acid-, heat-, and trypsin-resistant thus it is not broken down during food processing (1, 75).

The emetic syndrome is characterized by vomiting and nausea about 4 to 6 hours after ingestion. The duration of the illness is approximately between 6-24 hours (76). This is seen most associated with the consumption of starch products, generally rice or noodle dishes (10). However, the mechanism of the emetic toxin remains unclear.

The Diarrheaol Toxin

The diarrheaol type of food poisoning is caused by complex enterotoxins during vegetative growth in the small intestine of the host (76, 77). There have been five different enterotoxins characterized: Haemolysin BL (Hbl) a three-component toxin, Nonhaemolytic enterotoxin (Nhe), Cytotoxin K (CytK), Enterotoxin T (BceT), and Enterotoxin FM (EntFM). The former three enterotoxins are produced by bacteria in the small intestine and have known to cause food poisoning (72, 78, 79). BceT and EntFM have not been associated with food poisoning.

Symptoms of the diarrheal syndrome are abdominal cramps and diarrhea seen approximately 12 hours after consumption. The food products involved in this syndrome are quite varied. Food poisoning cases have been found associated with spoiled meat, vegetables, soup, and dairy (80, 81). Duration of illness is approximately between 12-24 hours and occasionally occurring for several days (76).

## 1.7 *Bacillus anthracis*

*B. anthracis* is a gram-positive, aerobic, non-motile, rod-shaped bacterium. *B. anthracis* is the agent that causes the fatal bacterial infection, anthrax. *B. anthracis* endospores enter the body through abrasions in the skin, ingestion, or inhalation (82). Once the spores are in the body, it germinates into its vegetative, pathogenic, form. The major virulence factors are encoded by two virulence plasmids, pXO1 and pXO2.

### 1.7.1 Types of Infection

Cutaneous anthrax appears to be involved in the 5$^{th}$ and 6$^{th}$ plagues of Egypt according to the 9$^{th}$ chapter of Exodus (83). It is found to enter subcutaneously through cuts or abrasions. In the United States, cutaneous infection account for approximately 95 percent of all anthrax infections (84). The initial symptoms are a nondescript skin lesion that appears a few days after the infection. This lesion leads to a black ulcer with significant swelling. Cutaneous anthrax is curable and can be treated with antibiotics.

Ingestional anthrax is found in the gastrointestinal or the oropharynx tract. Symptoms are seen a few days after the ingestion of contaminated meat (85). These symptoms include fever and abdominal pain. Ingestional anthrax can be fatal and death results from the perforation of the intestine.

Inhalational anthrax is rare and obtained through spores that are dispersed airborne from contaminated animal products. It has long been considered a potential biological warfare agent (86). The most recent attack of air dispersed anthrax was in 2001 during the anthrax bioterrorism attacks. Once spores are inhaled, alveolar macrophages engulf the spores and are transported to the lymph nodes where they germinate (87). This allows the bacteria to multiply and spread throughout the body by accessing the bloodstream

(85). Initial symptoms resemble those of upper respiratory tract infections, such as signs of fever and cough. Even with aggressive antimicrobial therapy, this type of anthrax is mostly fatal.

### 1.7.2 Toxins

The virulence factors of *B. anthracis* are the exotoxin protein and the poly-D-glutamic acid capsule that are encoded by two virulence plasmids, pXO1 and pXO2, respectively. The pXO1 plasmid is required for the production of the exotoxins, and pXO2 is required for the synthesis of the capsule. For virulence to occur, both plasmids must be present (88).

The exotoxin is composed of three proteins, the edema factor (EF), lethal factor (LF), and the protective antigen (PA). The three toxin components combine to form two binary toxins, the lethal toxin and the edema toxin (89). The lethal toxin is comprised of the LF and PA, and the edema toxin is comprised of EF and PA. PA binds to the cell surface receptor and mediates the entry of LF and EF into the host cell (85). LF is a zinc metalloprotease that cleaves members of the MAPKK family. This cleavage leads to apoptosis of the macrophages and release of tumor necrosis factor α and interleukin-1β, involved in host death (88). EF is a calcium-calmodulin dependent adenylyl cyclase that converts intracellular ATP into cAMP. Increased cAMP concentrations disrupt cellular water homeostasis, leading to membrane damage and necrosis. In addition, the edema factor causes edema subcutaneously (85). It has been suggested that the function of the exotoxins is to inhibit the immune response against the infection of *B. anthracis*.

The pXO2 plasmid contains the three genes necessary for the synthesis of the poly-D-glutamyl polypeptide. Capsule production is most important during the establishment of

the disease. The poly-D-glutamyl capsule protects the bacterium against anti-microbial components of serum and by inhibiting phagocytic engulfment of the vegetative bacilli (88).

# CHAPTER 2

# REVIEW OF RELATED LITERATURE

Germinants are specific amino acids or nucleosides that can trigger germination of bacterial spores. Germinants are highly diverse, among the *Bacillus* specie. This is due to the variety of germination receptors that can bind to the germinants and trigger germination.

## 2.1 *Bacillus cereus*

There has been two well studied strains of *B. cereus*, 3711 (ATCC 14579) and 569 (ATCC 10876). *B. cereus* ATCC 14579 has seven *ger* operons (*gerQ, gerG, gerK, gerL, gerS, gerI, gerR*). A study that used mutant strains in each of the *ger* operons (90) showed that the GerR receptor was involved in all responses with a single amino acid, except glutamine. The GerI receptor was involved in phenylalanine and inosine germination, whereas the *gerL*-deficient strain showed similar germination results to the wild type. *GerR, gerQ,* and *gerI* mutants showed reduced rates of germination with inosine, indicating that these receptors are involved in inosine signaling. The other four Ger receptors did not influence germination under the conditions used in that study (90).

*B. cereus* 569 has been shown to germinate in response to L-alanine and inosine, though germination can be initiated by L-alanine and inosine as a sole germinant (91). Three *gerA* operons involved in the germination response of L-alanine and inosine have been identified so far, *gerL, gerI,* and *gerQ* (92, 93). The *gerL* operon is the major contributor for the germination of *B. cereus* spores with L-alanine as a sole germinant. The *gerQ* operon is required for inosine germination as a sole germinant and has no role

in the L-alanine germination response. In the presence of L-alanine and inosine, the germination response was normal (92). The *gerI* operon responds to both the addition of L-alanine and inosine to trigger the germination response (93). Δ*gerI* spores were able to germinate in response to both L-alanine and inosine, however with inosine as a sole germinant, no germination response was triggered. When L-alanine was used as a sole germinant with Δ*gerI* spores, the rate of germination decreased. (93).

A few studies have suggested that receptors may be part of a complex that is involved with other *ger* operons that is necessary for nutrients to initiate the germination process (94, 95). In *B. subtilis,* there is genetic evidence for the physical interaction between the A, B, and C protein; GerAA interacting with GerAB, GerBA interacting with GerBB, and GerAC and GerBC interacting with their respective A and B proteins (96, 97). There are also studies that have suggested that GerBC protein may interact with GerAA-GerAB component of the *ger*A operon (97). In *B. cereus* 569, experiments that examine germination with inosine alone have shown that both GerI and GerQ receptors are required for its response (92, 93, 98). This indicates that only when the two receptors are complexed with inosine germination occurs.

By studying the germination kinetics of *B. anthracis* and *B. cereus*, the mechanism of how germinant bind to its receptor and the effects of the germinants on its cooperative nature may be suggested.

## 2.2 *Bacillus anthracis*

*B. anthracis* spores require either a combination of an amino acid and a nucleoside or two different amino acids to germinate (94). *B. anthracis* has seven *ger* operons*; gerA, gerH, gerK, gerL, gerS, gerY and gerX.* The GerX receptor function is unknown and is encoded on the pX01 plasmid (39, 99).

*B. anthracis* germination receptors and their germinants have been well studied by P. Hanna and colleagues (43). A germination model based on *B. anthracis* was proposed, which suggest that receptors interact in five distinct pathways leading to germination (43, 94). Each of the pathways requires at least two receptors for germination to occur.

The model illustrates four distinct amino acid and nucleoside-dependent germination pathways (see Table 1). The fifth pathway is the L-alanine pathway, which triggers *B. anthracis* spore germination at concentrations higher than 30mM (43). Two of these pathways require L-alanine and another amino acid. Treatment of *B. anthracis* spores with L-alanine and L-proline requires the coordinated activation of the GerL and GerK receptors. On the other hand, treatment with L-alanine and aromatic amino acids requires the GerS, GerH, and GerL receptor

Table 1.Germinant combinations in *B. anthracis*.

| | | Second Germinant | | |
|---|---|---|---|---|
| | | Inosine | L-alanine | |
| First Germinant | L-valine, L-serine | GerL, GerH, GerS or GerX | --- | Ger receptors |
| | L-methionine | GerK, GerH, GerS or GerX | --- | |
| | L-proline | GerK, GerH, GerS or GerX | GerL, GerK | |
| | L-histidine | GerH, GerS | GerH, GerS, GerL | |
| | L-tyrosine, L-tryptohan, L-phenylalanine | GerH, GerS | GerH, GerS, GerL | |

Combinations of amino acids and nucleoside that are involved with the indicated Ger receptors. Adapted from Fisher, N., Hanna, P. (2005)

The other two pathways require a purine nucleoside, inosine, and an amino acid. Treatment of *B. anthracis* spores with inosine and L-alanine, L-serine, L-valine, L-methionine, or L-proline requires the coordinated activation of various combinations of the GerL, GerH, GerK, GerS, and GerX receptors. When inosine is combined with either L-histidine or aromatic amino acids *B. anthracis* spore germination requires the coordinated activation of the GerH and GerS receptors.

## 2.3 Hypothesis

We propose that by applying mathematical approaches to spore germination, we can determine whether the binding of one germinant to the spore receptor will affect the binding of another germinant to another receptor. This implies that the corresponding Ger receptors must interact to alter their respective binding sites. We thus pursued this approach in two different species. In *B. cereus*, we concentrated on the inosine germination response to understand cooperative binding of a single germinant. In *B. anthracis*, we explored all possible germination pathways to understand how different

pathways interact. With *B. cereus* Δ*GerI* and Δ*GerQ* mutants, both inosine and L-alanine binding showed cooperativity. With *B. anthracis*, varied cooperativity, including positive and negative cooperativity, was seen between the different germinant combinations.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Bacteria strains, Plasmids, and Media

The *Bacillus cereus* strain used in this study is *B. cereus* 569 (ATCC 10876), obtained from America Type Culture Collection (Manassas, VA). The *Bacillus anthracis Sterne 34F2* strain was a generous gift from Dr. Arturo Casadevall from the Albert Einstein College of Medicine. The Sterne strain lacks the pXO2 plasmid. The Δ*GerI* (AM1314, Tn917-LTV1::*gerIA*5 (ino-5) $Ery^r$ Trp-1 $Str^r$) strain and Δ*GerQ* (AM1311, Tn917-LTV1::*gerQA*2 (ino-2) $Ery^r$ trp-1 $Str^r$) was a generous gift from Anne Moir (University of Sheffield, UK). All samples were prepared with ultrapure water. All amino acids were purchased from Sigma Aldrich Corporation (St. Louis, MO), of the highest purity available.

## 3.2 Spore Preparation

*Bacillus cereus* - *B. cereus* cells were plated on LB agar. Individual colonies were grown in LB (Luria-Bertani) broth at 37°C for 3-4 hours at ~90rpm. Cells were spread on LB agar to obtain a bacterial lawn. *B. cereus* plates were incubated at 37° for 3 days. Spores were then collected by flooding with ice-cold deionized water. Spores were harvested by centrifugation (Beckman J2-HS) for 5 minutes at 8000 rpm at 4°C. They were then re-suspended in ice-cold water at 8000 rpm for 5 minutes. The spores were purified by using a 20-50% Histodenz gradient and subjected to centrifugation at 11500 rpm at 4°C for 35 minutes. Supernatant is removed and the spores were washed five times with ice-cold water and stored at 4°C (98).

*Bacillus anthracis* - *B. anthracis* cells were plated on nutrient agar (20g of agar per liter) supplemented by 10% KCl, 1.2% $MgSO_4$, 1M $Ca(NO_3)_2$, 0.01M $MnCl_2$, and 1mM $FeSO_4$. Individual colonies were grown in LB (Luria-Bertani) broth at 37°C for 3-4 hours at ~90rpm. Cells were replated on LB agar to obtain a bacterial lawn. *B. anthracis* plates were incubated at 37° for 5 days. Spores were collected as described for *B. cereus* by flooding with ice-cold deionized water. Spores were harvested by centrifugation (Beckman J2-HS) for 5 minutes at 8000 rpm at 4°C. They were then re-suspended in ice-cold water and re-suspending it in ice-cold water at 8000 rpm for 5 minutes. The spores were purified by using a 20-50% Histodenz gradient and subjected to centrifugation at 11500 rpm at 4°C for 35 minutes. Supernatant is removed and the spores were washed five times with ice-cold water and stored at 4°C (98).

### 3.3 Germination Kinetic Assay

Because spores have a highly dense structure, they scatter light strongly. Upon germination the spore core hydrates, which reduces light scattering. This property was used to measure germination by following the decrease in optical density at 580 nm ($OD_{580}$) of a spore suspension after exposure to germination conditions. *Bacilli* spores were heat-activated at 70°C for 30 minutes. This step inactivates all vegetative cells. The spores were re-suspended in germination buffer (50mM Tris-HCl pH 7.5, 10mM NaCl) twice and a finally re-suspended to an $OD_{580}$ of 1.0 as measured in a Biomate 5 spectrophotometer. The suspension was monitored for auto-germination for 20 minutes. All germination experiments were carried out with non-germinated spores.

Experiments were carried out in a 96-well plate (200μL) using Labsystems iEMS 96-well plate reader equipped with a cut-off 540nm filter (ThermoElectron Corporation, Waltham, MA). Experiments were done in triplicates. Optical density readings were taken in 1 minute intervals for one hour at 25°C.

The data obtained were plotted to express the relative OD as a fraction of each individual $OD_{580}$ divided by the initial $OD_{580}$. Germination rates were calculated as the slope of the linear portion of the relative OD vs. time. The resulting data were plotted as a double reciprocal plot, 1/v vs. 1/[germinant concentration], to obtain $K_m$ (the spores' affinity to the substrate) and $V_{max}$ (maximum rate of germination). Binding constants for spores that exhibited cooperativity are indicated as *K'* which is an apparent binding constant that contains both $K_m$ in the presence of saturating concentration of substrate and the interacting factors that are involved in the cooperativity between the different binding sites. A Hill plot was graphed to determine the Hill number, n, which corresponds to the minimum number of germinant binding sites. (See figure 4 for a general scheme of the plots, see figure 5 for a general scheme describing cooperativity between the germinants.)

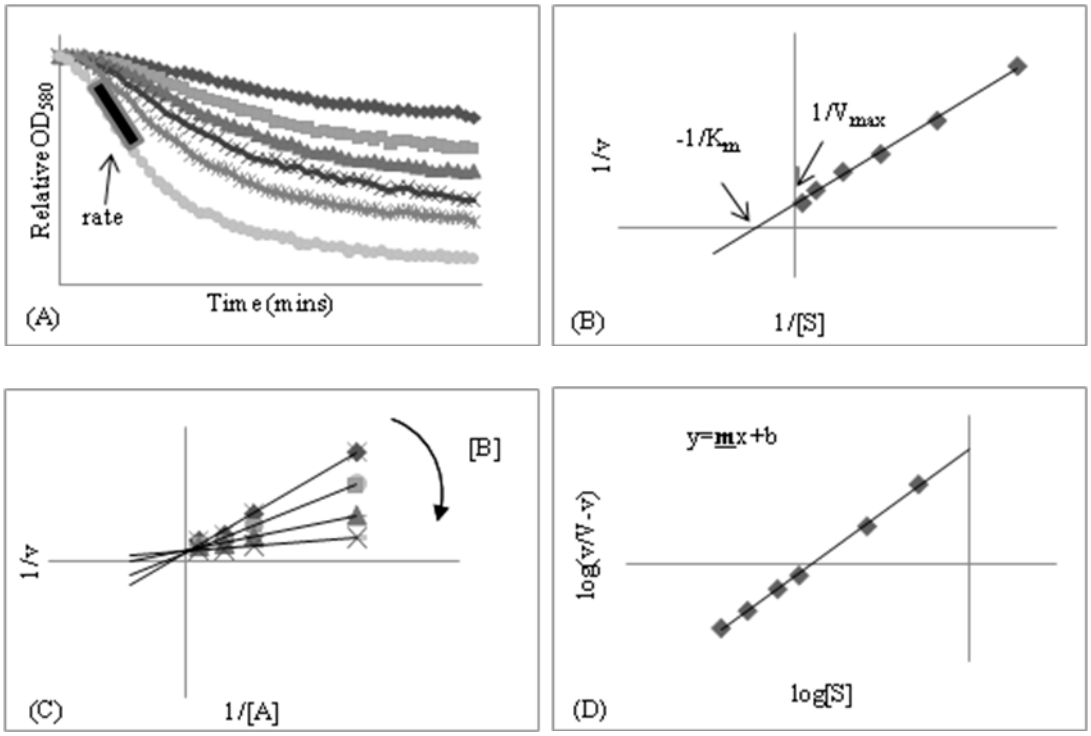



Figure 4. General schematic of plots. (A) Relative OD data over time, where the rate can be calculated by the slope of the linear region of each line. (B) A diagram of the double reciprocal plot where the $K_m$ and $V_{max}$ can be obtained. (C) The addition of increasing concentration of the germinant B as germinant A is treated as the substrate. (D) A Hill plot where m will indicate the Hill number.

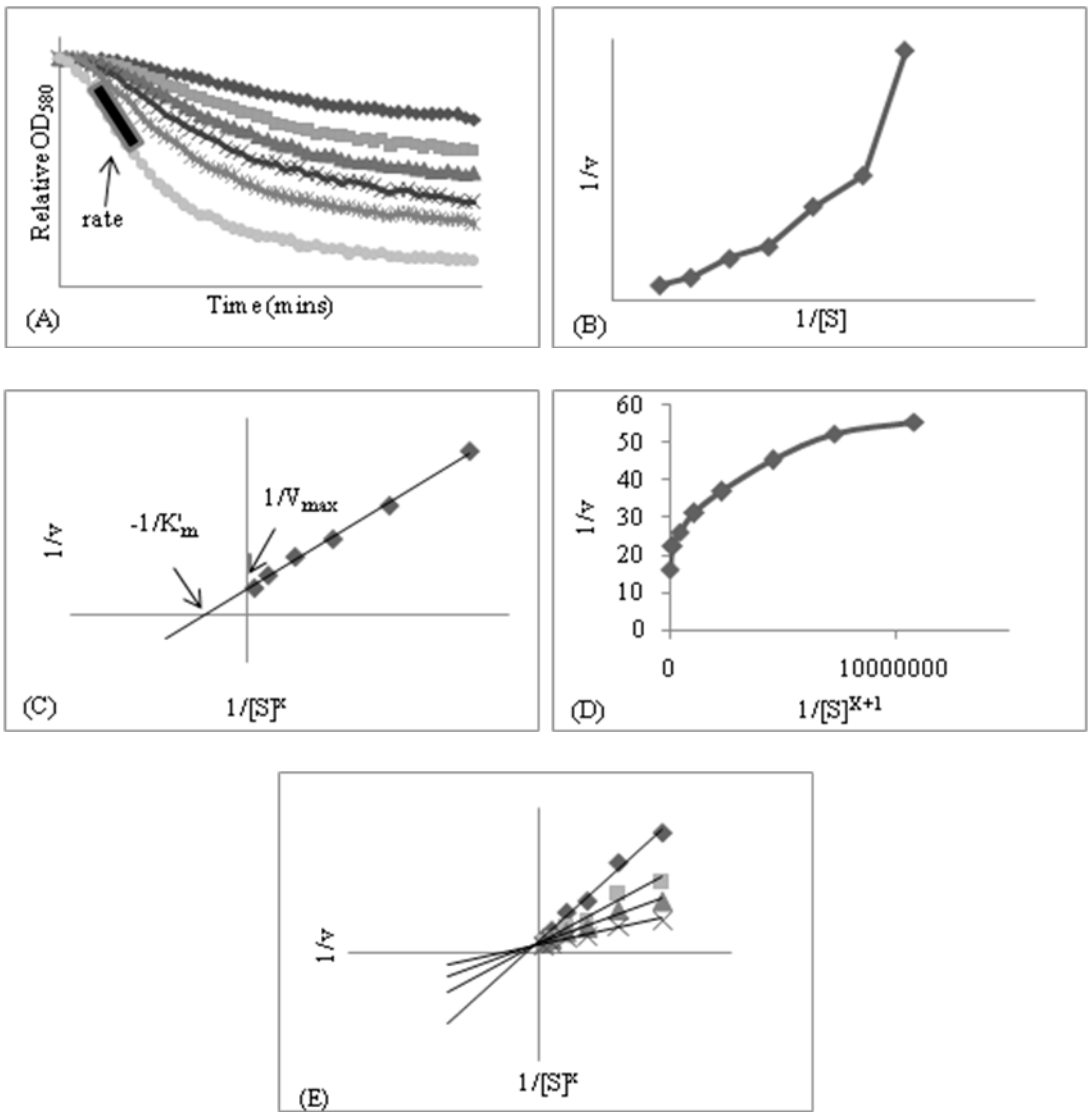



Figure 5. Schematic of plots showing cooperativity. (A) General relative OD over time plot that allows for the calculation of the rate. (B) A double reciprocal plot, 1/rate vs 1/[substrate] with curved line, an indication of cooperativity. (C) A double reciprocal plot, 1/v vs $1/[substrate]^x$, with the x-axis substrate concentration brought to exponential power, x. The K' is indicated rather than the $K_m$ due to the interacting factors seen with cooperativity. (D) A double reciprocal plot, 1/v vs $1/[substrate]^{X+1}$ with curved lines. (E) A double reciprocal plot showing increasing concentrations of the constant germinant.

### 3.3.1 *Bacillus cereus* germination assay

Spores obtained from Δ*gerI* were subjected to germination with variable inosine concentrations (0.0021, 0.0024, 0.0028, 0.0035, 0.0045, 0.006, 0.009, 0.015 mM), at different constant L-alanine concentrations (0.0325, 0.05, 0.075, and 0.1 mM). These concentrations were chosen to spread the data points in the double reciprocal plots. Concomitantly, spores were also subjected to germination with variable L-alanine concentrations (0.002, 0.004, 0.006, 0.008, 0.01, 0.012, 0.014, 0.016 mM) at different constant inosine concentrations (0.025, 0.05, 0.075, 0.01 mM).

Spores obtained from Δ*gerQ* were subjected to germination with variable inosine concentrations (0.0021, 0.0024, 0.0028, 0.0035, 0.0045, 0.006, 0.009, 0.015 mM) at different constant L-alanine concentrations (0.0325, 0.05, 0.075, 0.1 mM). Concomitantly, spores were also subjected to germination with variable L-alanine concentrations (0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009 mM) at different constant inosine concentrations (0.025, 0.05, 0.075, 0.1 mM).

### 3.3.2 *Bacillus anthracis* germination assay

Germination of *B. anthracis* spores with inosine and L-valine:

Spores were subjected to germination with variable inosine concentrations (0.01, 0.015, 0.02, 0.03, 0.045, 0.075, 0.2, 0.7 mM), at different constant L-valine concentrations (0.0325, 0.075, 0.25, 0.5 mM). Concomitantly, spores were also subjected to germination with variable L-valine concentrations (0.005, 0.0075, 0.01, 0.025, 0.05, 0.1, 0.5, 1.5 mM) at different constant inosine concentrations (0.0325, 0.05, 0.075, 0.1mM).

Germination of *B. anthracis* spores with inosine and L-serine:

Spores were subjected to germination with variable inosine concentrations (0.02, 0.035, 0.05, 0.075, 0.1, 0.25, 0.5, 1.0 mM), at different constant L-serine concentrations (0.01, 0.05, 0.1, 0.5 mM). Concomitantly, spores were also subjected to germination with variable L-serine concentrations (0.0075, 0.01, 0.04, 0.1, 0.4, 0.9, 1.5 mM) at different constant inosine concentrations (0.0325, 0.075, 0.125, 0.2 mM).

Germination of *B. anthracis* spores with inosine and L-methionine:

Spores were subjected to germination with variable inosine concentrations (0.02, 0.03, 0.05, 0.07, 0.09, 0.1, 0.2, 0.3, 0.5 mM), at different constant L-methionine concentrations (0.01, 0.02, 0.06, 0.09 mM). Concomitantly, spores were also subjected to germination with variable L-methionine concentrations (0.02, 0.03, 0.04, 0.06, 0.09, 0.15, 0.3, 0.6 mM) at different constant inosine concentrations (0.02, 0.025, 0.03, 0.05 mM).

Germination of *B. anthracis* spores with inosine and L-histidine:

Spores were subjected to germination with variable inosine concentrations (0.03, 0.04, 0.05, 0.065, 0.08, 0.2, 0.35, 1mM), at different constant L-histidine concentrations (0.0075, 0.01, 0.025, 0.05 mM). Concomitantly, spores were also subjected to germination with variable L-histidine concentrations (0.0075, 0.01, 0.015, 0.025, 0.04, 0.06, 0.08, 0.2 mM) at different constant inosine concentrations (0.025, 0.035, 0.05, 0.075 mM).

# CHAPTER 4

# FINDINGS OF STUDY

## 4.1 *Bacillus cereus*

Two inosine receptors have been reported on *B. cereus* 569 and elimination of either of the two receptors can disable inosine-mediated germination. However, *B. cereus* 569 spores with a mutated inosine receptors can still germinate in the presence of inosine when supplemented with L-alanine (93). It was suggested that L-alanine response can involve two loci, *gerI* and another loci (93) and inosine response was suggested to show cooperativity between two binding sites (98). In this study, *B. cereus* ΔGerI and ΔGerQ spores were treated with a combination of inosine and L-alanine and as expected, strong synergy was seen with both inosine and L-alanine. The resulting data for germinating spores were analyzed by fitting the change in germination rate to mathematical approaches in enzyme kinetics and the resulting values are shown in Tables 2 and 3.

### 4.1.2 *B. cereus* ΔGerI spores germinated with inosine and L-alanine

When *B. cereus* ΔGerI spores were treated with varying concentrations of L-alanine at different fixed concentrations of inosine, both the double reciprocal plot of 1/v vs. 1/[L-alanine] resulted in a family of upwardly curved plots rather than the expected linear plots. This is characteristic of positive allosteric cooperation (100). Similar plots were seen for the germination of wild type *B. cereus* spores when treated with inosine alone (98). The Hill plot also showed positive cooperativity with a Hill number of approximately 2.0. Double reciprocal plots of 1/v vs. $1/[\text{L-alanine}]^2$ at increasing inosine concentration resulted in linear plots, as expected (See figure 6). This suggests that binding an L-alanine molecule increases the affinity for binding of another L-alanine

molecule on a separate site. The plots of 1/v vs. 1/[L-alanine]$^2$ at increasing inosine concentration converged to the left of the y-axis, indicating that the maximum germination rate increases with increasing inosine concentrations. Similarly, the affinity of spores for L-alanine increases with increasing inosine concentrations. (Refer to Appendix I).

When *B. cereus* ΔGerI spores were treated with varying concentrations of inosine at different fixed concentrations of L-alanine, the double reciprocal plot of 1/v vs. 1/[inosine] also resulted in a family of upwardly curved plots. Double reciprocal plot of 1/v vs. 1/[inosine]$^2$ at increasing L-alanine concentrations results in linear plots, indicating positive cooperativity (See figure 6). The Hill plot also showed positive cooperativity with a Hill number of approximately 1.7. The double reciprocal plots, in contrast to L-alanine binding, converged at the y-axis indicating that inosine does not affect the maximum germination rate, but spores' affinity for inosine increases with increasing L-alanine concentrations. (The cooperativity plot seen in both inosine and L-alanine binding is similar to figure 5). (Refer to Appendix I).

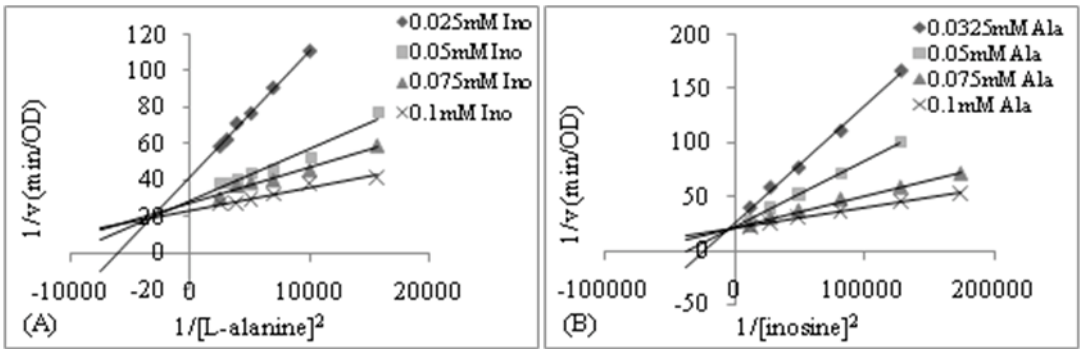



Figure 6. *B. cereus* Δ*GerI* germination with inosine + L-alanine. (A) Double reciprocal plot of titrating L-alanine concentration with constant inosine concentration. (B) Double reciprocal plot of titrating inosine concentration with constant L-alanine concentration.

Table 2. Kinetic parameters for *B. cereus* Δ*GerI*

| Germinant | Hill number (n) | K' (uM) | $V_{max}$ (OD/hr) |
|---|---|---|---|
| L-alanine | 2.0 | 2.3x10-2 | 3.5x10-2 |
| Inosine | 1.7 | 5.1x10-6 | 4.7x10-2 |

*K'* is an apparent binding constant that contains both the $K_m$ of the substrate and the interacting factors that are involved in the cooperativity between the different binding sites.

4.1.3 *B. cereus* ΔGerQ spores germinated with inosine and L-alanine

When *B. cereus* ΔGerQ spores were treated with varying concentrations of L-alanine at different fixed concentrations of inosine, both the double reciprocal plot of 1/v vs. 1/[L-alanine] resulted in a family of upwardly curved plots rather than the expected linear plots. This is characteristic of positive allosteric cooperation (100). The Hill plot also showed positive cooperativity with a Hill number of approximately 2.5. Double reciprocal plots of 1/v vs. $1/[\text{L-alanine}]^2$ at increasing inosine concentration results in linear plots, as expected (See figure 7). This suggests that binding an L-alanine molecule increases the affinity for binding of another L-alanine molecule on a separate site. The plots of 1/v vs. $1/[\text{L-alanine}]^2$ at increasing inosine concentration converged to the left of the y-axis, indicating that the maximum germination rate changes with increasing inosine concentrations. Similarly, the affinity of spores for L-alanine increases with increasing inosine concentrations. (Refer to Appendix II).

When *B. cereus* ΔGerQ spores were treated with varying concentrations of inosine at different fixed concentrations of L-alanine, the double reciprocal plot of 1/v vs. 1/[inosine] also resulted in a family of upwardly curved plots. Double reciprocal plot of 1/v vs. $1/[\text{inosine}]^2$ at increasing L-alanine concentrations results in linear plots, indicating positive cooperativity (See figure 7). The Hill plot also showed positive

cooperativity with a Hill number of approximately 1.9. The double reciprocal plots, in contrast to L-alanine binding, converged at the y-axis indicating that inosine does not affect the maximum germination rate, but spores' affinity for inosine increases with increasing L-alanine concentrations. (The cooperativity plot seen in both inosine and L-alanine binding is similar to figure 5). (Refer to Appendix II).

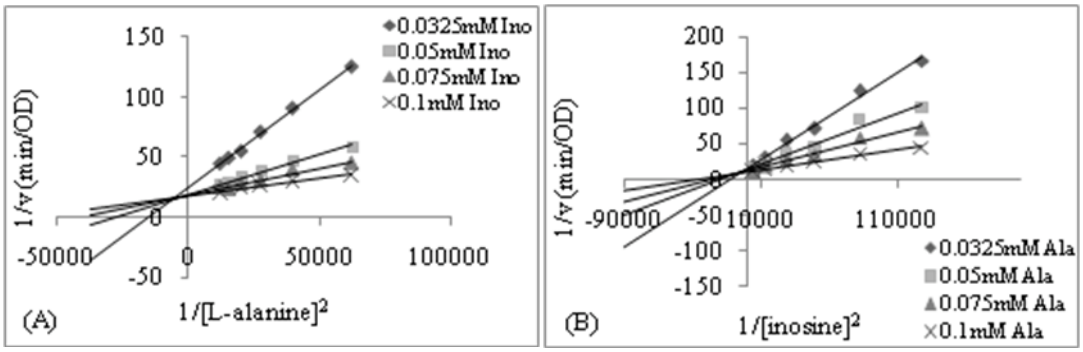



Figure 7. *B. cereus* Δ*GerQ* germination with inosine + L-alanine. (A) Double reciprocal plot of titrating L-alanine concentration with constant inosine concentration. (B) Double reciprocal plot of titrating inosine concentration with constant L-alanine concentration.

Table 3. Kinetic parameters for *B. cereus* Δ*GerQ*

| Germinant | Hill number (n) | K' (uM) | $V_{max}$ (OD/hr) |
|---|---|---|---|
| L-alanine | 2.5 | 4.8x10-4 | 4.9x10-2 |
| Inosine | 1.9 | 8.70x10-3 | 7.5x10-2 |

*K'* is an apparent binding constant that contains both the $K_m$ of the substrate and the interacting factors that are involved in the cooperativity between the different binding sites.

## 4.2 *Bacillus anthracis*

*B. anthracis* spores were treated with a combination of different amino acids and nucleosides. The combinations that exhibited decrease in OD within physiological

concentrations are inosine/L-valine, inosine/L-serine, inosine/L-methionine, and inosine/L-histidine. Combinations that failed to induce germination are inosine/L-proline, inosine/all aromatic amino acids, L-alanine/L-proline, and L-alanine/all aromatic amino acids with concentrations above 10mM. The resulting data for germinating spores were analyzed by fitting the change in germination rate to Michaelis-Menten equations and the resulting values obtained in shown in Table 1.

Table 4. Kinetic parameters for *B. anthracis;* indicating $K_m$, $V_{max}$, and Hill number

| Germinant | Hill number (n) | $K_m$ (uM) | $V_{max}$ (OD/min) |
|---|---|---|---|
| L-valine | 0.9 | 2.9 | 4.0x10-2 |
| Inosine | 1.3 | 47.4 | 7.1x10-2 |
| | | | |
| L-serine | 0.4 | *7.3 | 8.5x10-2 |
| Inosine | 1.0 | 30.9 | 4.8x10-2 |
| | | | |
| L-methionine | 1.1 | 2.1 | 3.3x10-1 |
| Inosine | 2.1 | *1.2 | 4.6x10-2 |
| | | | |
| L-histidine | 0.9 | 2.4 | 5.6x10-2 |
| Inosine | 1.5 | 24.3 | 4.3x10-2 |
| | | | |
| †L-alanine | ND | ND | ND |
| †Inosine | ND | 270 | 4.0x10-2 |

*indicates *K'*, a constant that contains both the $K_m$ of the substrate and the interacting factors that are involved in the cooperativity between the different binding sites. Inosine + L-phenylalanine; inosine + L-proline; L-alanine + L-phenylalanine; L-alanine + L-tyrosine; L-alanine + tryptophan; L-alanine + L-proline showed little to no germination at 10mM. †indicates data obtained from M. Akoachere and colleagues, 2007. ND: not determined

### 4.2.1 *B. anthracis* spores germinated with inosine and L-valine

When *B.* anthracis spores were treated with varying concentrations of L-valine at different fixed concentrations of inosine both the double reciprocal plot and Hill number

suggest that L-valine binds to its cognate receptor, following classical Michaelis-Menten approximations. The double reciprocal plot for L-valine at different concentrations of inosine yielded a family of plots that converged at the y-axis. The convergence of plots at the y-axis, shows that the maximum germination rate ($V_{max}$) does not change with increasing inosine concentrations. Similarly, since each plot intersects the x-axis at a different position; it indicates that the affinity of spores for L-valine increases with increasing inosine concentrations.

Similarly, *B.* anthracis spores were treated with varying concentrations of inosine at different fixed concentrations of L-valine. Both the double reciprocal and the Hill plot suggest no allosteric activity in inosine binding. The double reciprocal plot also resulted in a convergence of the plots on the y-axis. Similar to inosine binding, L-valine affects the affinity of spores for inosine. This suggests that binding of each germinant alter the affinity of the other for spore binding. (Refer to Appendix II).

### 4.2.2 *B. anthracis* spores germinated with inosine and L-serine

When *B. anthracis* spores were treated with varying concentrations of L-serine at different fixed concentrations of inosine, both the double reciprocal plot of 1/v vs. 1/[L-serine] resulted in a family of downwardly curved plots rather than then expected linear plots. This is characteristic of a system with negative allosteric cooperation (100). The Hill plot also showed negative cooperativity with a Hill number of 0.4 (101). Double reciprocal plots of 1/v vs. $1/[\text{L-serine}]^{0.4}$ at increasing inosine concentration resulted in linear plots as expect. This suggests that binding an L-serine molecule reduces the affinity for binding of another L-serine molecule on a separate site. The plots of 1/v vs. $1/[\text{L-serine}]^{0.4}$ at increasing inosine concentration converged at the y-axis, indicating that

inosine does not affect the maximum germination rate, but does affect the affinity of spores for L-serine (See figure 8).

Similarly, *B.* anthracis spores were treated with varying concentrations of inosine at different fixed concentrations of L-serine. The double reciprocal plots were linear and the Hill plot had a slope of 1. The data suggests that in contrast to L-serine binding, binding of inosine shows no allosteric binding. Similar to L-serine binding, the family of double reciprocal plots converged at the y-axis, indicating that L-serine does not affect the maximum germination rate, but spores' affinity for inosine increases with increasing L-serine concentrations. (Refer to Appendix II).

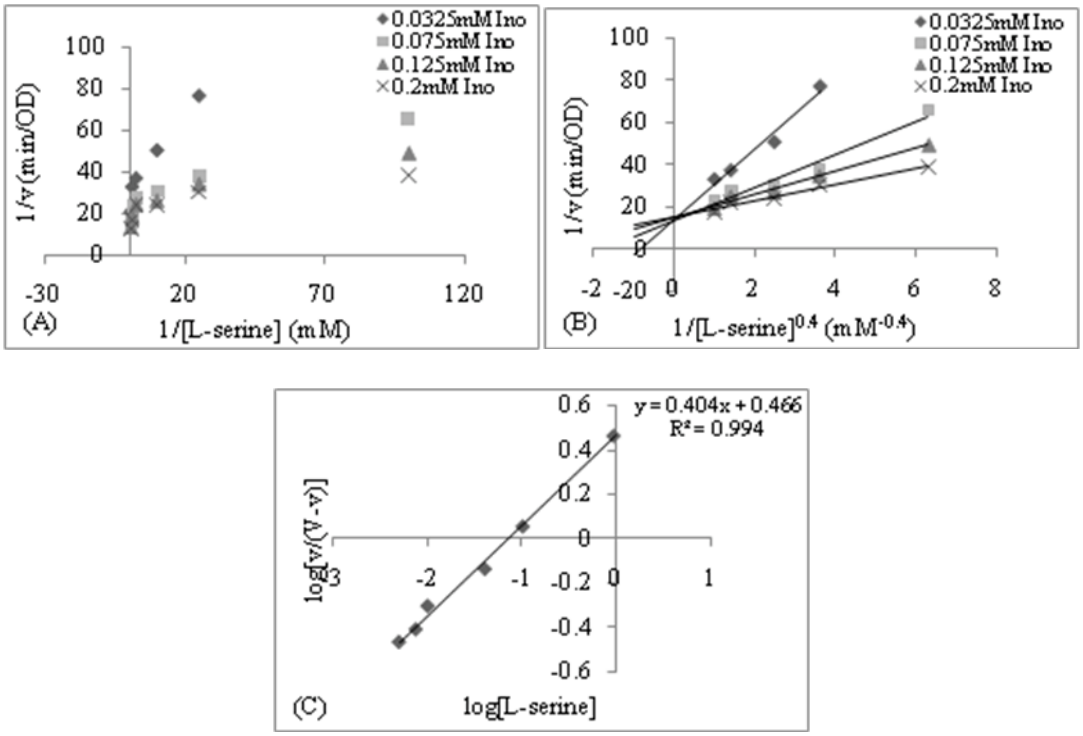



Figure 8. *B. anthracis* germination with inosine + L-serine. The hill plot is 0.2mM Ino. (A) Double reciprocal plot resulting in a family of curved plots, indicating cooperativity. (B) Double reciprocal plot with the substrate, L-serine, taken to the exponential power of 0.4 resulted in a family of linear lines. (C) Hill plot at 0.2mM inosine with Hill number approximately 0.4.

### 4.2.3 *B. anthracis* spores germinated with inosine and L-methionine

When *B.* anthracis spores were treated with varying concentrations of L-methionine at different fixed concentrations of inosine, both the double reciprocal plot and Hill plot suggest that L-methionine binds to its cognate receptor, following classical Michaelis-Menten approximations. The double reciprocal plot for L-methionine at different concentrations of inosine yielded a family of plots that converged to the left of the y-axis. This convergence to the left of the y-axis shows that the maximum germination rate changes with increasing inosine concentrations. Similarly, the affinity of spores for L-methionine increases with increasing inosine concentrations.

Similarly, when *B.* anthracis spores were treated with varying concentrations of inosine at different fixed concentrations of L-methionine, the double reciprocal plot of 1/v vs. 1/[inosine] resulted in a family of upwardly curved plots rather than the expected linear plots. Data suggests that, in contrast to L-methionine binding, binding of inosine shows positive allosteric cooperation. The Hill plot also showed positive cooperativity with a Hill number of approximately 2 (See figure 9). Double reciprocal plots of 1/v vs. $1/[\text{inosine}]^2$ at increasing L-methionine concentrations results in linear plots, as expected. This suggests that binding an inosine molecule increases the affinity for binding of another inosine molecule on a separate site. The plots of 1/v vs. $1/[\text{inosine}]^2$ at increasing L-methionine concentrations converged at the y-axis, indicating that inosine does not affect the maximum germination rate, but spores' affinity for inosine increases with increasing L-methionine concentrations. (Refer to Appendix II).

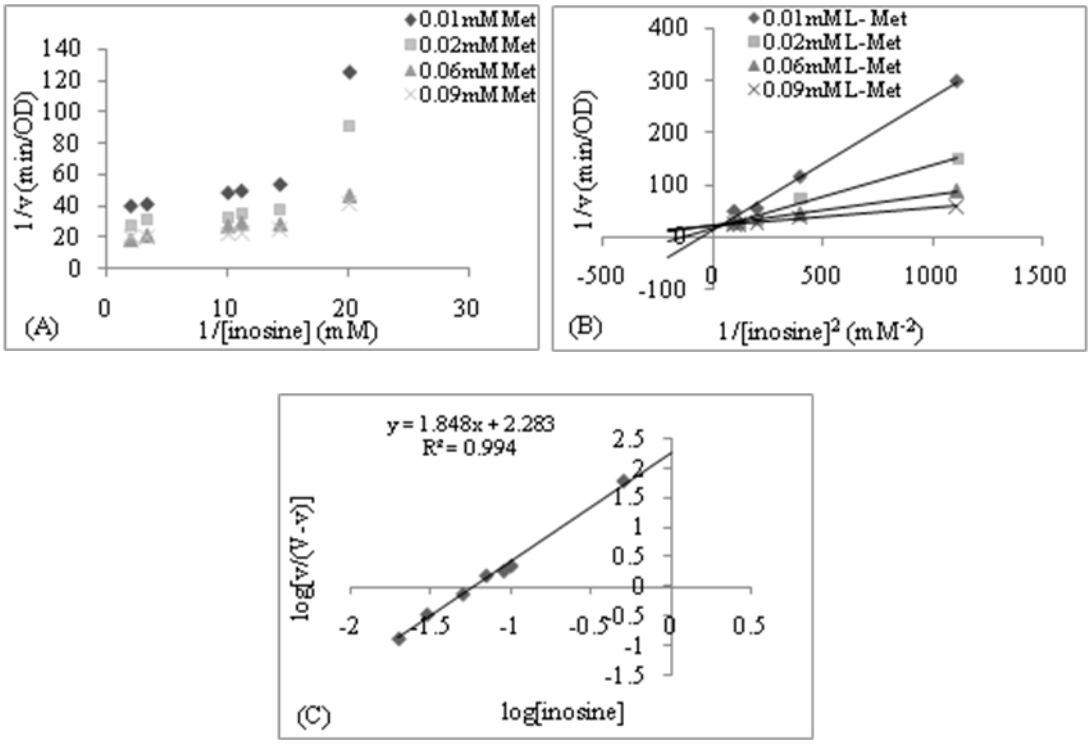



Figure 9. *B. anthracis* germination with inosine + L-methionine. (A) Double reciprocal plot resulting in a family of curved plots, indicating cooperativity. (B) Double reciprocal plot with the substrate, inosine, squared resulted in a family of linear lines. (C) Hill plot at 0.09mM L-methionine, with Hill number approximately 2.

### 4.2.4 *B. anthracis* spores germinated with inosine and L-histidine

When *B.* anthracis spores were treated with varying concentrations of L-histidine at different fixed concentrations of inosine both the double reciprocal plot and Hill number suggest no allosteric activity in L-histidine binding. The double reciprocal plot resulted in a convergence of linear plots to the left of the y-axis. This convergence to the left of the y-axis shows that the maximum germination rate changes with increasing inosine concentrations. Similarly, the affinity of spores for L-histidine increases with increasing inosine concentrations.

Similarly, *B.* anthracis spores were treated with varying concentrations of inosine at different fixed concentrations of L-histidine. The double reciprocal plots were linear and the Hill plot had a slope of 1, suggesting that there is no allosteric activity inosine binding. The double reciprocal plot resulted in a convergence of linear plots at the left of the y-axis, showing that the maximum germination rate does not change with increasing L-histidine concentrations, as the affinity of spores for inosine increasing with increasing L-histidine concentrations. (Refer to Appendix II).

The germination kinetics between the substrates inosine and L-proline; L-alanine and L-proline; inosine with the aromatic acids (L-phenylalanine, L-tyrosine, and L-tryptophan); and L-alanine with the aromatic amino acids showed no significant germination at the physiological concentration range of the substrates.

# CHAPTER 5

# SUMMARY AND CONCLUSIONS

The main objectives of this study were to determine the kinetic parameters for spore germination of *B. cereus* and *B. anthracis*. In turn, the data obtained can be used to deduce Ger receptor interactions and to propose plausible mechanistic model for germinant binding.

## 5.1 Discussion of *Bacillus cereus* Results

*B. cereus* ΔGerI and ΔGerQ spores did not germinate in the presence of inosine unless supplemented with L-alanine. According to E. Abel-Santos and T. Dodatko, 2007, the kinetic pathway of *B. cereus* 569 exhibited complex and strongly cooperative characteristics with inosine binding. It was suggested that there are two inosine binding sites, one from each of the GerI and GerQ receptor (See figure 10). To initiate the process of germination inosine would need to bind to both the binding sites on the GerI and GerQ receptor. This led us to study the germination kinetics of *B. cereus* ΔGerI and ΔGerQ spore strains with inosine and L-alanine to infer the Ger receptor interactions.


Figure 10. Scheme of allosteric cooperativity: two different Ger protein receptors.

The kinetic analysis of the germination of both the mutants (Δ*gerI* and Δ*gerQ*) revealed that the germination process show significant synergy between binding sites of the same protein receptor. The GerI receptor is involved in inosine and L-alanine germination (93) and the GerQ receptor is involved in the germination of inosine as a sole germinant (92). When inosine was treated as the substrate in experiments involving both the mutants, synergistic effects were still seen. It was previously suggested that the interaction of inosine binding to one receptor (e.g. *gerI*) would increase the affinity for inosine binding at a second receptor (e.g. *gerQ*) (98). This present data, in contrast to previous suggestions propose that the binding of inosine at one protein receptor will increase the affinity for inosine binding at another of the same protein receptor (See figure 11).

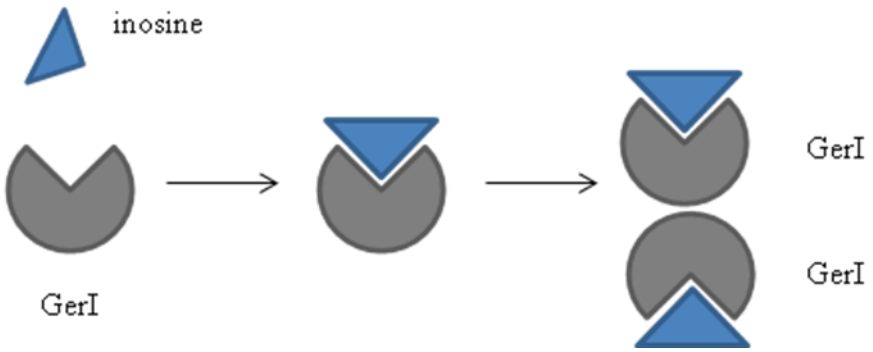



Figure 11. Scheme of allosteric cooperativity: two same Ger protein receptors. This type of cooperativity can be suggested for L-alanine binding to the GerL receptor in our present study.

Data supporting the synergistic effect with Δ*gerI* and Δ*gerQ* mutant is indicated in the double reciprocal plot of 1/v vs. $1/[\text{inosine}]^2$ and from the Hill number being approximately 2. Since both mutants were able to show synergistic effects without the

presence of the other receptor, it strongly suggests the binding of the substrate to one protein receptor will cause the affinity of that substrate to bind to another same protein receptor. This suggests that with the Δ*gerI* mutant, inosine would bind to the binding site on the GerI receptor and increase its affinity for inosine binding to another binding site on another GerI receptor in order to germinate. In the Δ*gerQ* mutant, inosine would bind to the binding site on the GerQ receptor and increase its affinity for inosine binding to another binding site on another GerQ receptor.

Based off the present data, this synergistic activity is also seen for L-alanine interaction with the germination receptors. The GerI receptor is involved in inosine and L-alanine germination (93) and the GerL receptor is involved in the germination of L-alanine as a sole germinant (92). When L-alanine was treated as the substrate in experiments involving both the mutants, synergistic effects were seen. This present data suggests that the binding of L-alanine at the binding site of one protein receptor will increase the affinity for L-alanine binding at another binding site of the same protein receptor.

All data support the germination kinetics in a sequential manner where both substrates must bind to the binding sites prior to the initiation of germination. The double reciprocal plot can suggest a mechanism for the binding of the substrate to its receptor. The Δ*gerI* mutant suggests a system where this combination of germinant follows the sequential ordered mechanism, whereby L-alanine binds to the binding site before inosine can bind to initiate germination. The Δ*gerQ* mutant suggests a system similar to the Δ*gerI* mutant where it follows the sequential ordered mechanism, having L-alanine binding to the binding site prior to inosine in order to initiate the process of germination.

Both *B. cereus* 569 and ATCC14579 have members of GerI, GerQ, and GerL germination receptors, the GerI receptor show high levels of similarity between the two strains (90). The GerI receptor is involved in inosine and L-alanine germination with *B. cereus* 569. *B. cereus* ATCC14579 has a GerR receptor that is also involved in a germination response with inosine and L-alanine (44). It was shown that inosine and L-alanine mediated germination cannot be compensated with any other germination receptors. The GerR receptor seems to be highly involved in the response with inosine and L-alanine while this present study suggests that the GerI and GerQ receptor both individually responds to inosine and L-alanine. The GerQ receptor seems to respond to inosine in *B. cereus* ATCC14579, however the germination characteristics of this receptor remains unclear. It is unclear why this response differs between the two *B. cereus* strains. Thus, it seems that *B. cereus* 569 does not contain a similar GerR receptor, but the process of germination can be initiated with inosine and L-alanine in the presence of only the GerI or the GerQ receptor.

## 5.2 Discussion of *Bacillus anthracis* Results

Spore germination is the first step to *B. anthracis* pathogenicity. Particular combination of germinants can cause efficient germination, leading to the anthrax disease. We have previously shown that the germination of *B. anthracis* spores with inosine and L-alanine showed synergy between the two substrates (102). We determined the kinetics of the germination pathways triggered by all combinations of germinant pairs that have been reported to be sufficient for *B. anthracis* spore germination.

*B. anthracis* spores were subjected to germination with a diverse set of amino acids and nucleosides. Our results show that certain germinant combination that have been reported to induce *B. anthracis* spore germination showed no significant effect at physiologically relevant concentrations. This implicates that the concentrations in the host is negligible in comparison to the concentrations needed to initiate germination in this study. We infer, thus, that germination with the germinant combinations that shown no significant effect at physiologically relevant concentrations would not germinate in the host. This in vitro analysis has identified the apparent affinity of the germinants to their receptor, rate of maximum germination, and the number of binding sites. Dependent on its germinant combination, kinetic studies with specific amino acids and nucleosides showed variations in their binding effects. All the experiments indicated that for each germinant pair the spore's affinity for one substrate increases with an increasing concentration of the second germinant.

Our studies indicate that the same receptors could show either positive or negative cooperativity, depending on the germinants used for activation. Combinations of inosine/L-serine and inosine/L-valine all activate the GerL, GerH, and GerS or GerX receptors. Negative cooperativity was seen when the GerL, GerH, and GerS or GerX receptor was activated with L-serine. In contrast, inosine/L-valine shows no cooperative nature. The GerL is required for both L-serine and L-valine germination with inosine (43). The GerH receptor, on the other hand, has been shown to be involved in the purine nucleoside recognition (103). Thus, the differences in the cooperative nature and non-cooperative nature of L-serine and L-valine may be due to the effects of the chemical structure of the substrates on the GerL receptor, suggesting that it is not differences in the

receptor itself that causes different germination responses. The inosine/L-valine, inosine/L-serine combination involved with the GerL, GerH, and GerS or GerX receptors show a sequential random mechanism. This type of mechanism indicates that both substrates must bind in random order, before germination can occur (See figure 12). This type of system is similar to our previous report on the germination with inosine and L-alanine (102).

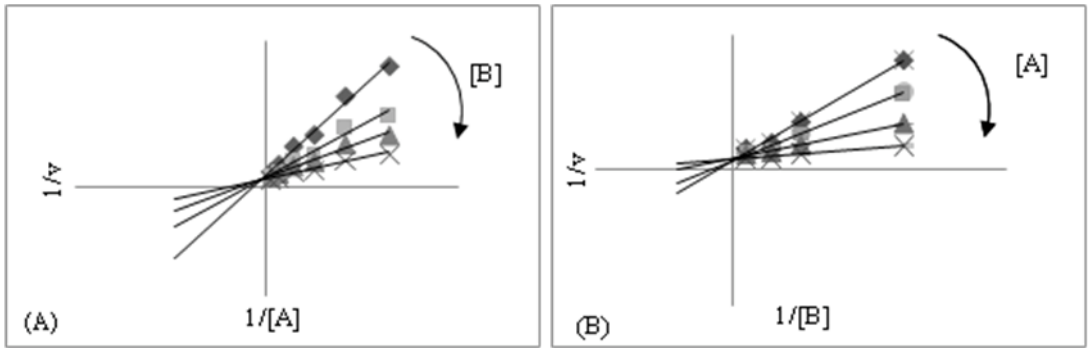



Figure 12. Schematic for sequential random mechanism. (A) At increasing B concentrations and titrating substrate A, the plot intercepts on the y-axis. (B) At increasing A concentrations and titrating substrate B, the plot also intercepts on the y-axis. This type of system suggests a sequential random mechanism.

Kinetic analysis revealed that positive cooperativity is seen with the GerK, GerH, and GerS or GerX receptors with inosine at given constant L-methionine concentrations following the sequential ordered mechanism. These synergistic effects were also seen with *B. cereus* in the involvement of inosine as a sole germinant (98). The cooperative nature of the individual receptors that interact is not clear. However, it can suggest that the binding of inosine will increase the spores' affinity of inosine to the next binding site. The GerK receptor is required for both the germination of L-proline and L-methionine

with inosine, both combinations use the GerK, GerH, and GerS or GerX receptors (43). The differences seen in the effects of the insignificant germination of L-proline in physiological conditions as oppose to L-methionine remains unclear. It remains to be shown why certain germinants causes particular cooperative effects as others do not. In contrast to the amino acid/nucleoside combination involving the GerL, GerH, and GerS or GerX receptors, the inosine/L-methionine combination involving the GerK, GerH, and GerS or GerX receptors show a sequential ordered mechanism. This type of mechanism indicates that inosine binds to the binding site before L-methionine can bind to initiate the process of germination (See figure 13).

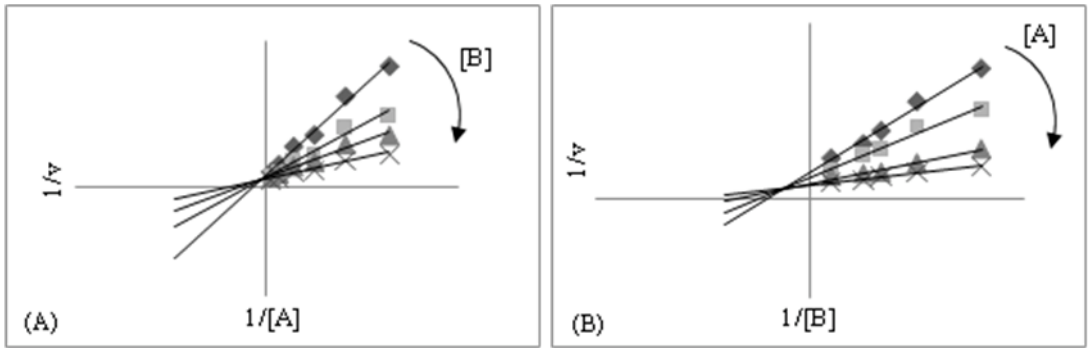



Figure 13. Schematic for sequential ordered mechanism. (A) At increasing B concentrations and titrating substrate A, the plot intercepts on the y-axis. (B) At increasing A concentrations and titrating substrate B, the plot intercepts to the left of the y-axis, on the first quadrant. This type of system indicates a sequential ordered mechanism.

The germinant combination inosine and L-histidine showed no cooperative nature, but did suggest a sequential ordered mechanism. This combination involves the GerH and GerS receptors. It has been shown that GerH receptor is involved in the recognition of L-

histidine and inosine, with an absolute requirement for inosine and no other purine nucleoside (103). The GerS receptor has been shown to respond to aromatic ring structures (94). It has also been shown that the null GerH and null GerS receptors showed similar germinant profiles thus suggesting that the two may be redundant (103). Thus specific receptor recognition of L-histidine is not yet clear. The effects of inosine with the aromatic amino acids with the GerH and GerS receptors, shows no significant germination at physiological substrate concentrations. The differences seen in the receptor recognition to the germinant between L-histidine and the aromatic amino acids remains unclear. Similar to the inosine/L-methionine combination involving the GerK, GerH, and GerS or GerX, the inosine/L-histidine combination involving GerH and GerS show a sequential ordered mechanism. This type of mechanism indicates that L-histidine binds to the binding site before inosine can bind to initiate the process of germination.

All the cyclic compounds tested besides L-histidine (L-proline and aromatic amino acids: L-tryptophan, L-tyrosine, and L-phenylalanine) showed no significant germination at physiological substrate concentrations. This includes L-proline in combination with inosine and in combination with L-alanine. At higher than physiological conditions, germination was seen. Past studies have shown that few combinations of the germinants have showed roughly up to 56% of germination within the first 15 minutes with physiological concentrations (94). When the combination of L-proline and L-alanine were subjected to germination at concentration ranging up to 30mM, the rate of germination seen was significantly comparable to the rate of germination seen with L-alanine alone. This suggests that the germination seen at such high concentration ranges with this combination of germinants may be due to the effects of L-alanine alone. Due to

the many variable conditions that could affect germination, it can also be suggested that the purification process of the endospores could be a factor in the inconsistency of the results. This in vitro analysis established the individual $K_m$ and $V_{max}$ values of each germinant combination, as well as the suggested cooperative nature of those combinations.

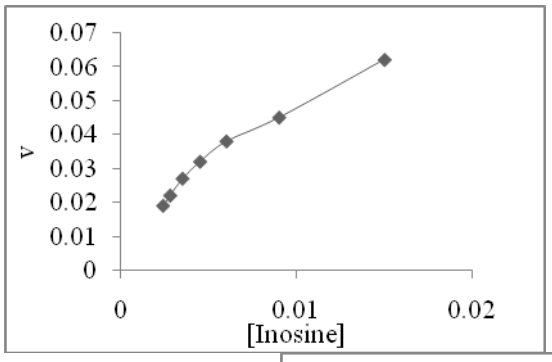
## 5.3 Conclusion and Recommendations for further Study

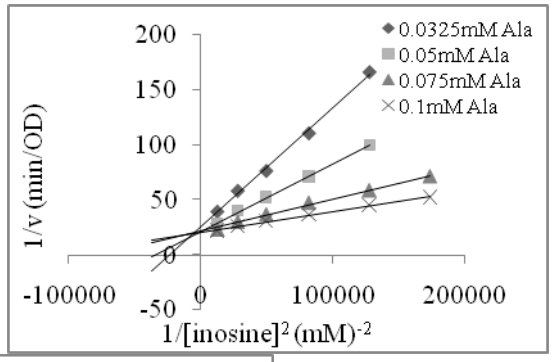
In conclusion, germination kinetics can determine the spores' affinity to its germinant and maximum rate of germination. These kinetic parameters allowed for a proposed mechanistic model for germinant binding. The binding sites on the receptors and its germinant specific tendencies seem to vary widely amongst the genus *Bacillus*. *B. anthracis* spore germination with inosine and amino acid (L-serine and L-methionine) seem to show a varied cooperativity, negative and positive, respectively. The reasons why different forms of cooperativity seen amongst the same set of Ger receptors remains unclear. The kinetic parameters found in this study can be used to compare it with macrophage concentrations of the amino acids and nucleosides to suggest a mechanism for the germination profile of *B. anthracis* spores. *B. cereus* spore germination has shown strong synergy with inosine binding to the GerI and GerQ receptor. This study also showed strong synergy for L-alanine binding sites. This signifies same protein receptor interaction for inosine in order to initiate germination. With L-alanine binding, it can be inferred that two different proteins interact or same protein interactions occur. To further determine the plausible mechanistic model, mutants and double mutants should be furthered studied.

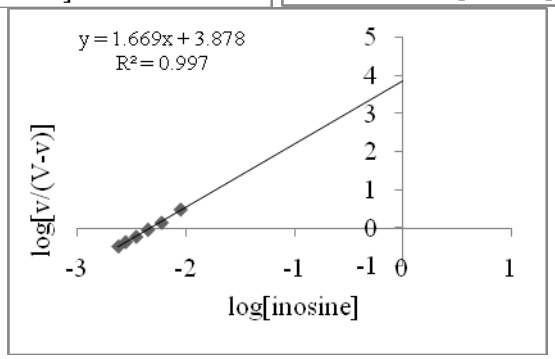
APPENDIX I

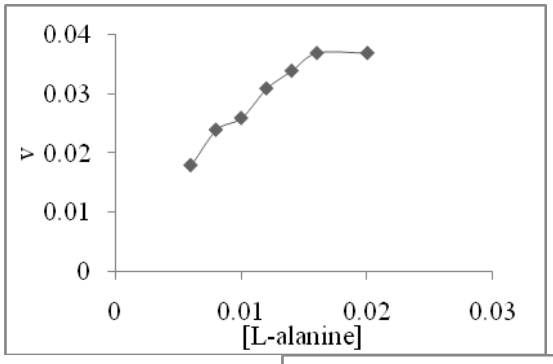
*BACILLUS CEREUS*

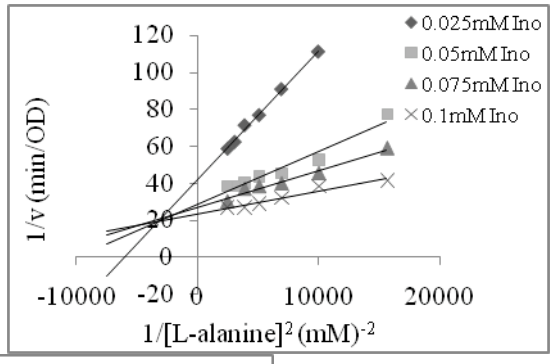
ΔGerI

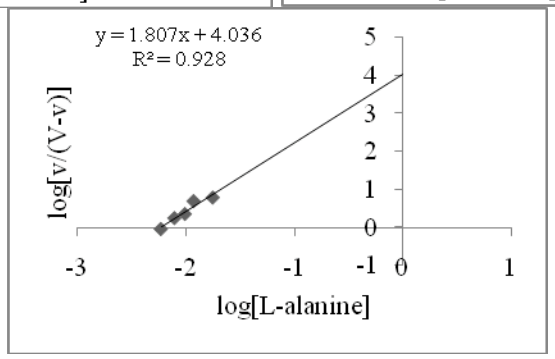


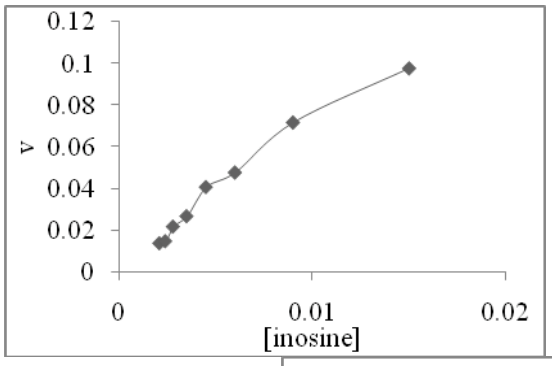



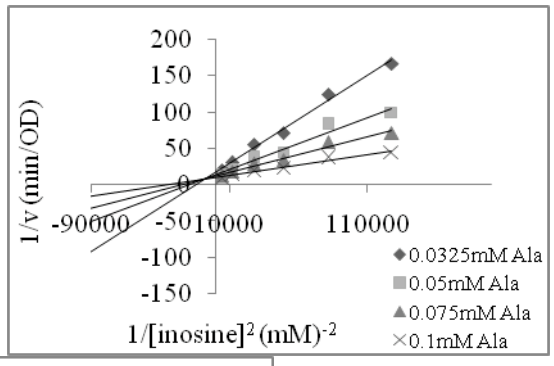



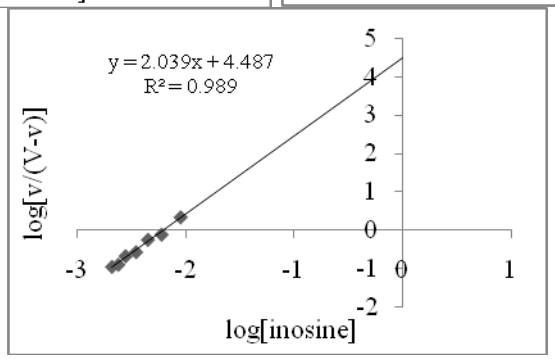



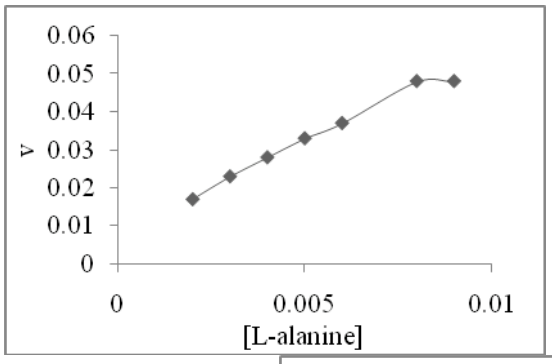



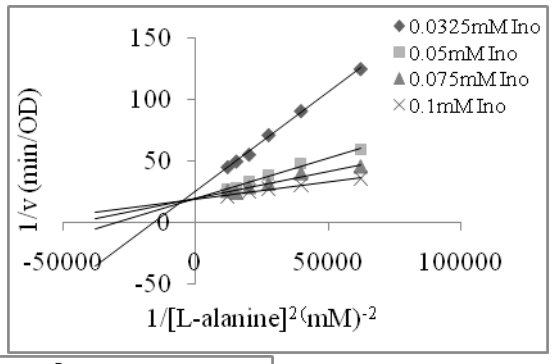
ΔGerQ

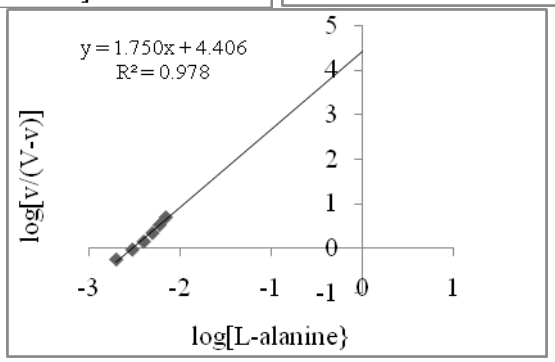

0.12
0.1
0.08
v 0.06
0.04
0.02
0
0
0.01
0.02
[inosine]


200
150
100
50
0
-90000
-50000
10000
110000
-100
-150
1/v (min/OD)
1/[inosine]2 (mM)-2
0.0325mM Ala
0.05mM Ala
0.075mM Ala
0.1mM Ala


y = 2.039x + 4.487
R² = 0.989
5
4
3
2
1
0
-3
-2
-1
-1
0
1
-2
log[v/(V-v)]
log[inosine]


0.06
0.05
0.04
v 0.03
0.02
0.01
0
0
0.005
0.01
[L-alanine]

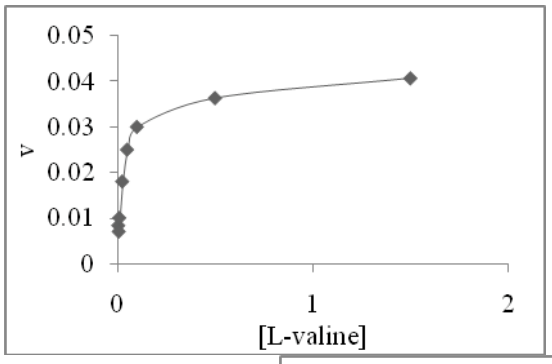

150
100
50
0
-50000
0
50000
100000
-50
1/v (min/OD)
1/[L-alanine]2 (mM)-2
0.0325mM Ino
0.05mM Ino
0.075mM Ino
0.1mM Ino

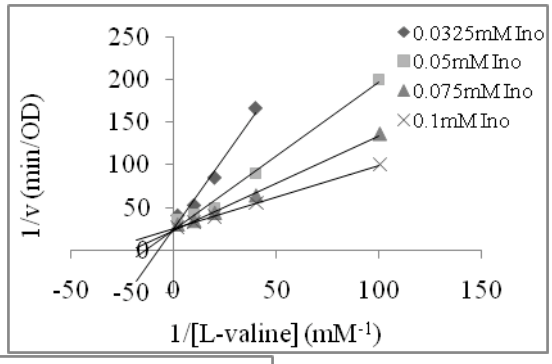

y = 1.750x + 4.406
R² = 0.978
5
4
3
2
1
0
-3
-2
-1
-1
0
1
log[v/(V-v)]
log[L-alanine}

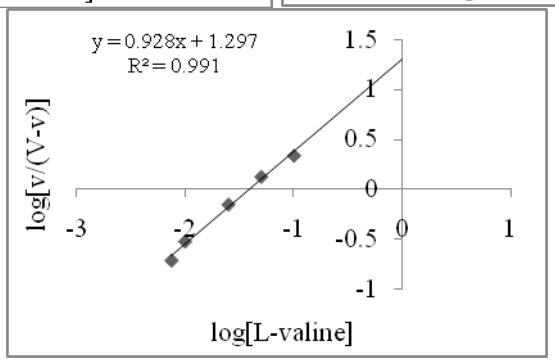
APPENDIX II

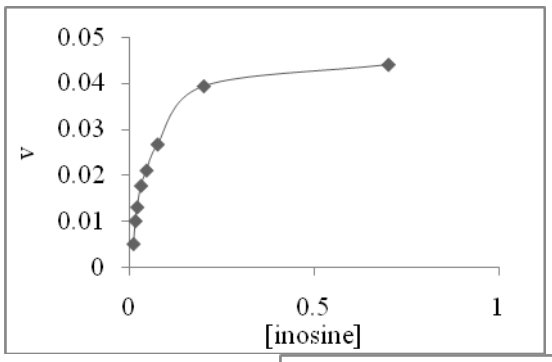
*BACILLUS ANTHRACIS*

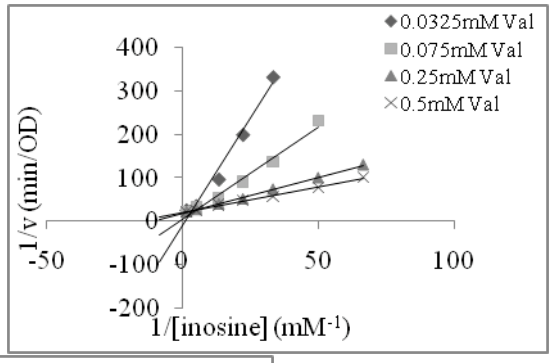
Inosine + L-valine

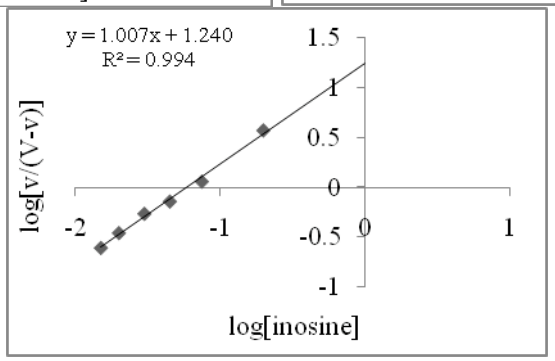


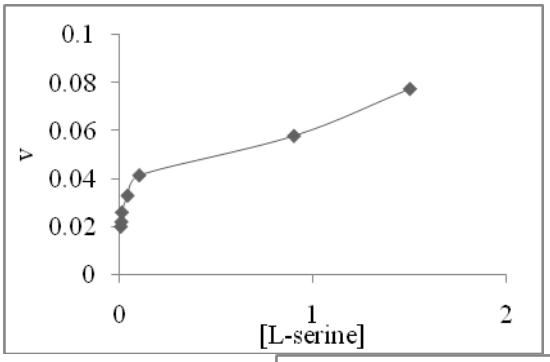



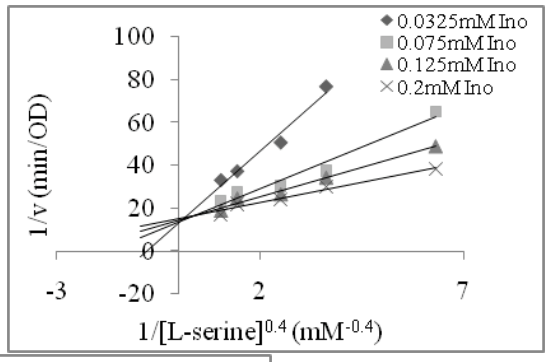



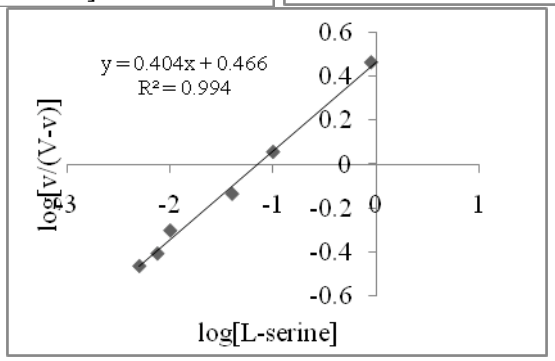



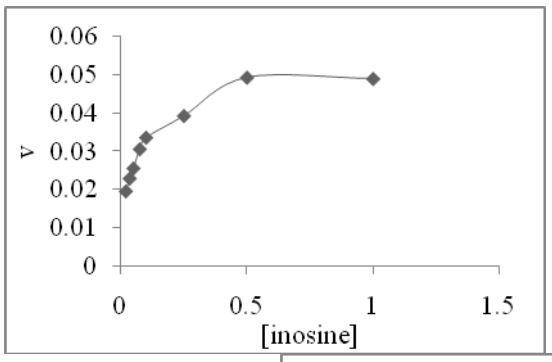



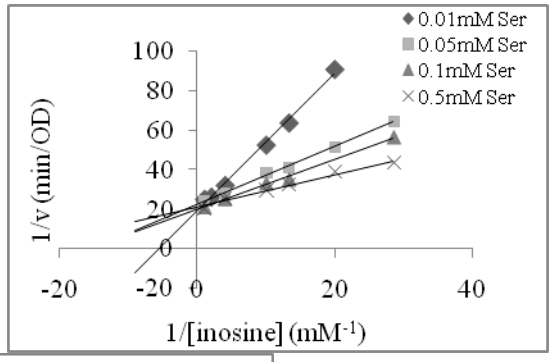
Inosine + L-serine

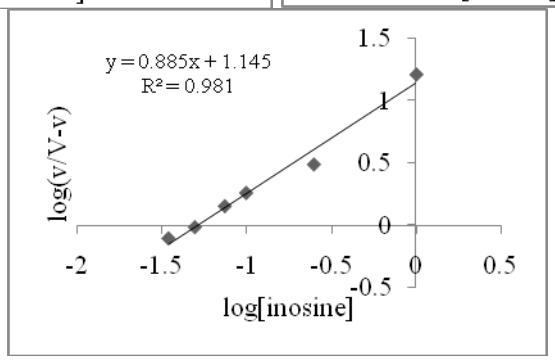


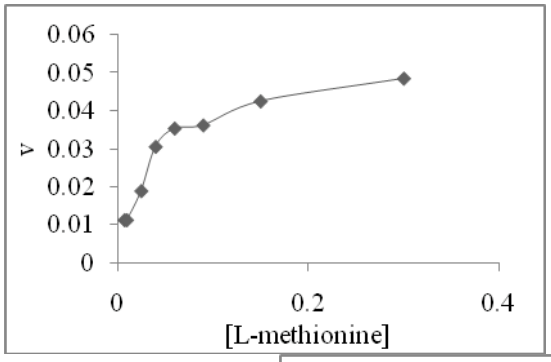



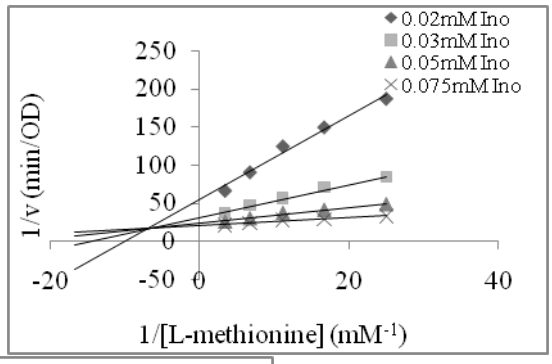



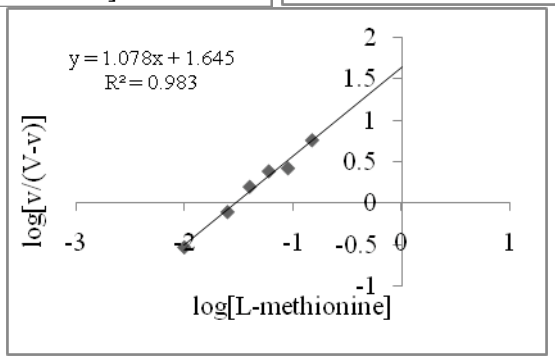



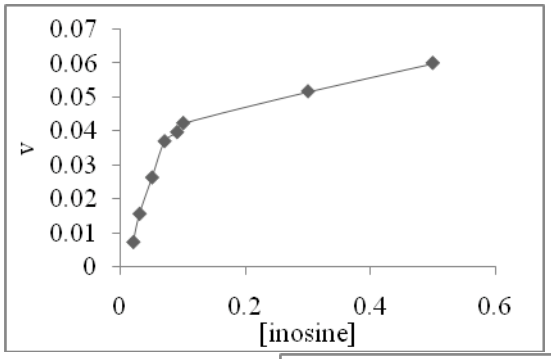



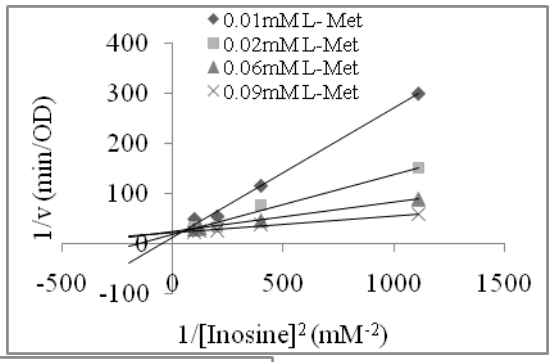
Inosine + L-methionine

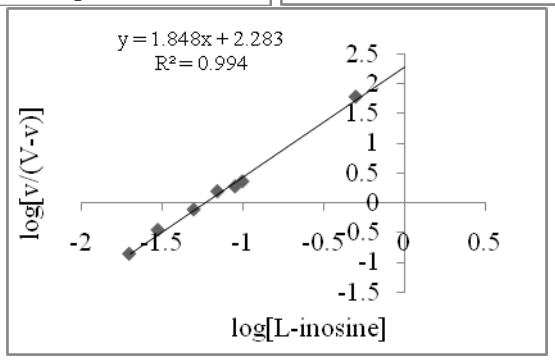


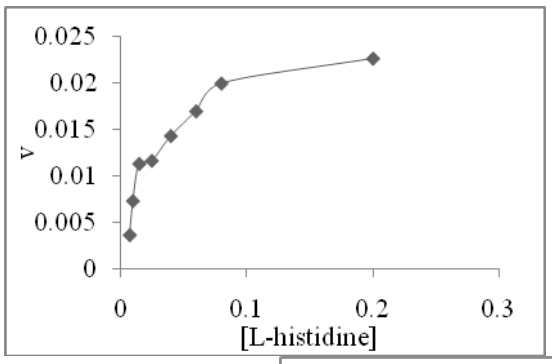



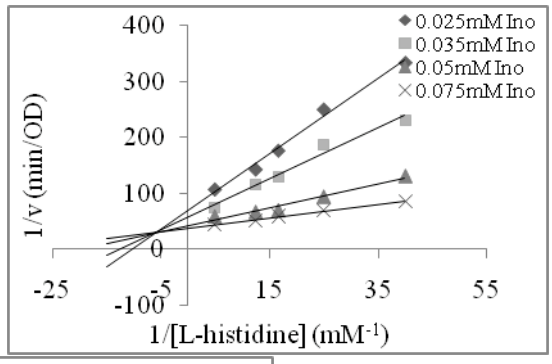



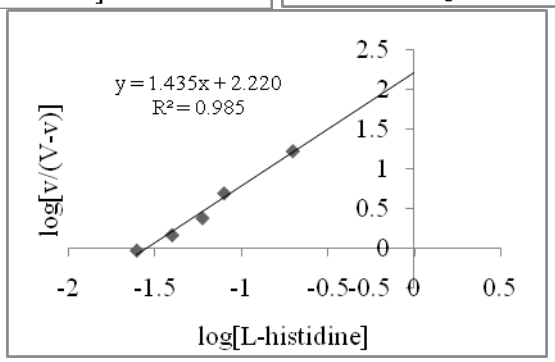



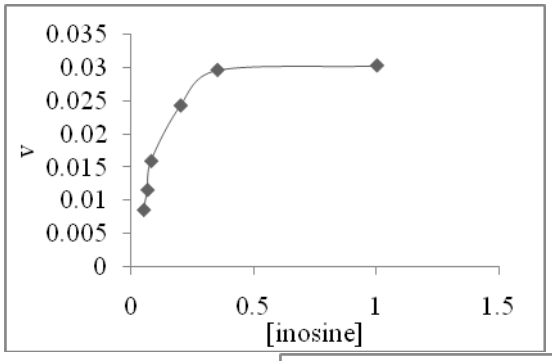



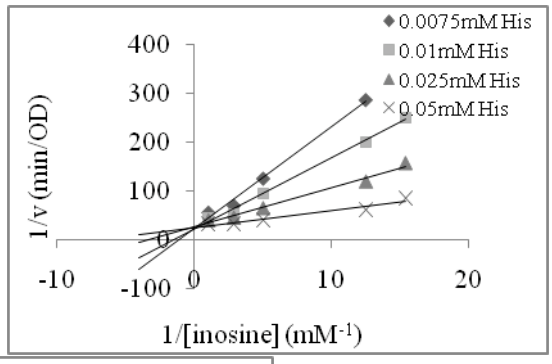
Inosine + L-histidine

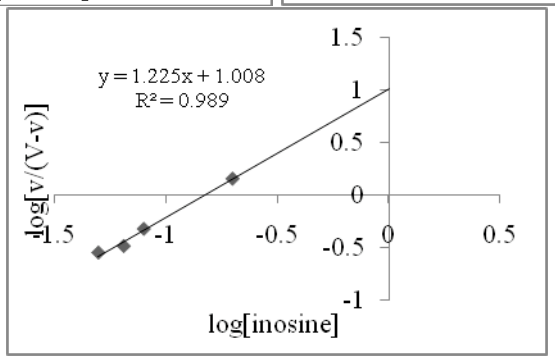


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